ROLE OF LEPTIN IN HIPPOCAMPAL SYNAPTOGENESIS

By

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ROLE OF LEPTIN IN HIPPOCAMPAL SYNAPTOGENESIS

Abstract

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Leptin, a 16KD peptide product of the obese (ob) gene, is a key regulator of food intake and energy homeostasis. In the brain, hypothalamus is considered the primary site for leptin activity and effects on feeding. However, leptin has been shown to affect hippocampal functioning and hippocampus-dependent behaviors such as spatial and emotion-related memory formation as well. Furthermore, intra-hippocampal leptin injection enhances memory retention and reduces depression in animal models for cognitive impairment and depression respectively. Abnormal hippocampal dendritic development and synaptogenesis are associated with cognitive and emotional disorders. In this dissertation, I explore the signaling mechanisms that mediate leptin’s effects on synapse formation in the hippocampus.

In chapter 1, I show that a transient receptor potential channel C (TrpC) current activated by acute leptin treatment is important for leptin-induced filopodia formation. Filopodia are potential immature synapses. Leptin activates the TrpC current by increasing membrane trafficking of TrpC channels via a CaMK pathway dependent activation of Rac1. The exact signaling molecules involved in this pathway are CaMKKβ, CaMKIγ, β-Pix and Rac1; these molecules mediate leptin-induced TrpC trafficking, activation and finally, filopodia formation.
In chapter 2, I focus on a cyclicAMP response-element binding protein (CREB)-dependent signaling mechanism that mediates leptin effects on glutamate synapse formation. CREB activity is required for glutamate synapse formation and it is known to mediate effects of other neurotrophic factors on synapse formation. Leptin induces dendritic spines and glutamate synapse formation in hippocampal neurons and leptin receptors (lepRs) mediate these effects both in vitro and in vivo. Activation of LepR leads to CREB phosphorylation via the Mek/Erk pathway resulting in initiation of CREB-dependent transcription of microRNA-132 (miR132), a well-known CREB target. Increase in expression of miR132 and suppression of p250GAP expression via miR132 is required for leptin-induced synapse formation.

In chapter 3, I report a non-CREB dependent mechanism for leptin-induced synapse formation. Kruppel-like factor4 (KLF4) is a transcription factor first identified in the gut and skin epithelium. Leptin treatment increases KLF4 mRNA and protein expression in vitro. I found that KLF4 overexpression increases glutamate synapse formation and KLF4 expression is required for leptin-induced synapse formation in hippocampal neurons.
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INTRODUCTION

Leptin, the peptide product of the obese (ob) gene is a well-known anorexigenic hormone that reduces food intake and increases energy metabolism. It increases with increasing fat mass and excessive leptin levels accompanied by leptin resistance are hallmarks of obesity. However, many studies over the years have found leptin to be a diverse regulator of various physiological functions beyond metabolism, body weight, and food intake. We now know that leptin serves brain development, immune function, reproduction, bone formation, bone growth, and cardiovascular functions among others (Ahima and Flier 2000).

Along with its peripheral effects, leptin has been shown to have many central nervous system effects including modulation of cognition and emotion-related behaviors. Low plasma leptin levels are correlated with age-related dementia and Alzheimer’s disease (Lieb, Beiser et al. 2009). Non-obese individuals with lower leptin levels have significantly higher chances of expedited cognitive decline leading to Alzheimer’s (Harvey 2010; Paz-Filho, Wong et al. 2010). Furthermore, leptin replacement therapy improves cognitive performance in leptin-lacking individuals with a null mutation in the leptin gene (Paz-Filho, Babikian et al. 2008). These cognition enhancing effects of leptin have been replicated in animal models that lack leptin production or signaling (Li, Aou et al. 2002; Takeda, Sato et al. 2010). Interestingly, in normal mice leptin administration enhances cognitive performance. Farr and colleagues found that intra-hippocampal leptin administration increases hippocampus-dependent spatial and fear memory retention in wild type mice as
well as SAMP8 transgenic mice, an animal model for Alzheimer’s (Farr, Banks et al. 2006).

Similar to its effects on cognition, leptin has been implicated in development of emotional disorders. Patients suffering from major depressive disorder have lower plasma leptin levels (Lu 2007). Furthermore, leptin-resistant obese individuals have higher chances of developing clinical depression (Lu 2007). In animal models of depression, plasma leptin levels drop below control levels and leptin replacement via intra-peritoneal injections improves their depression-like symptoms (Lu, Kim et al. 2006). Together these studies suggest an important role for leptin in adult brain functioning. It is important to note that leptin is also important for brain development and ‘programming.’ In rats, extremes in leptin exposure during embryonic or neonatal period affect metabolic as well as cognitive functioning during adulthood (Trottier, Koski et al. 1998; Ahima, Bjorbaek et al. 1999; Proulx, Clavel et al. 2001; Taylor and Poston 2006). Rats born to dames fed on a high fat diet tend to maintain a higher body weight and fat mass when compared to rats born to dames fed in a regular laboratory chow (Trottier, Koski et al. 1998; Taylor and Poston 2006). Also, mice lacking leptin, ob/ob, or the leptin receptor, db/db, have lowered expression of critical synaptic proteins such as syntaxin-1 and synaptobrevin in the neocortex and the hippocampus (Ahima, Bjorbaek et al. 1999). Together these studies present leptin as a valuable hormone to study due to its involvement in human health conditions such as age-related dementia, depression and developmental effects of excessive maternal leptin levels.

Beyond the impact on public health, understanding the role of leptin signaling in the brain regions important for cognition and emotions is an important step
towards understanding how peripheral signals such as hormones modulate complex behaviors. Hormones are chemical messengers released from one cell type that can affect the functioning of many other cell types. They carry information about changes in bodily functions in response to external or internal conditions. This information when supplied to other cell types allows them to respond appropriately to altered conditions. Therefore, it is plausible to expect this information to impact the brain’s cognitive machinery as well and influence adaptive behaviors. Receptors for many hormonal ligands are present in various brain regions. To name a few: leptin, ghrelin, cholecystokinin, insulin, estrogen, glucocorticoids, mineralocorticoids receptors are present in brain (Moult and Harvey 2008). Leptin’s effects on cognition and emotion could indicate the brain’s utilization of the leptin signal to create salient food-associated memories (Moran and Gao 2006). Food sources need to be located, evaluated and remembered for survival.

The hippocampus is an important brain region involved in declarative and spatial memory formation as well as emotional learning. Post-mortem studies have revealed a dramatic decrease in hippocampal volume in patients suffering from major depressive disorders (Duman and Monteggia 2006). Understanding the mechanisms behind leptin’s effects on hippocampal functioning could reveal how leptin modulates cognition and emotions.

In this dissertation, I focus on excitatory synapse formation in the hippocampus to understand leptin’s effects on hippocampal functioning. Excitatory synapses occur on actin-enriched, morphologically distinct protrusions on the dendritic shaft called dendritic spines (Harris and Kater 1994). Alterations in the number and shape of dendritic spines have been correlated with various psychiatric
disorders, such as schizophrenia and depression (Vaidya and Duman 2001; Nimchinsky, Sabatini et al. 2002). Additionally, a decrease in spine number is observed with age-related dementia and Alzheimer’s disease (Yu and Lu 2012). Synapse formation and maintenance are highly correlated with cognitive performance and emotional stability (Dierssen and Ramakers 2006; Pittenger and Duman 2008). Provided this information, dendritic spines seem like an important neural substrate for cognitive and emotional processing. Therefore, it is plausible that leptin effects on hippocampal function could be mediated via its effects on hippocampal dendritic spines. Here, I describe signaling mechanisms initiated by acute (Chapter 1) and chronic (Chapters 2 & 3) leptin treatment that alter synapse formation and maintenance in the hippocampus.

**Leptin**

Leptin is a key hormonal regulator of food intake. It is a 16kD polypeptide product of the obese (ob) gene located on chromosome 7 in humans. The obese gene mutation was first discovered in a yellow mice colony at the Jackson laboratories in 1949 (Ingalls, Dickie et al. 1951). The ob gene was later cloned in 1994 and leptin, its protein product, was isolated (Zhang, Proenca et al. 1994). The structure of leptin is highly homologous amongst different species. Leptin belongs to a family of helical cytokines and shares significant structural similarities with other members of this group (Madej, Boguski et al. 1995). Leptin, like other cytokines, contains an intra-chain disulphide bond required for its biological activity (Grasso, Leinung et al. 1997).
Leptin and Metabolism

The most well studied function of leptin is regulation of feeding behavior and body weight. Leptin null ob/ob mice are over-weight and have significantly higher food intake (Ingalls, Dickie et al. 1951). A homozygous frame-shift mutation in the ob gene in humans results in congenital obesity (Montague, Farooqi et al. 1997). The insatiable appetite of ob/ob mice can be controlled and brought down to normal levels by injecting them with leptin (intra-peritoneal or intra-cerebro-ventricular) (Campfield, Smith et al. 1995; Halaas, Gajiwala et al. 1995). The increased levels of plasma glucose and insulin found in the ob/ob mice can also be normalized by intra-peritoneal leptin injections (Pelleymounter, Cullen et al. 1995). The concentration of leptin circulating in the blood plasma was found to be directly proportional to the mass of white adipose tissue in the body (Maffei, Halaas et al. 1995). Therefore, leptin, which is synthesized and secreted primarily by white adipose tissue cells (Masuzaki, Ogawa et al. 1995), is thought to serve as an “adiposity signal”.

The effects of leptin on feeding behavior are regulated by various humoral factors. Leptin production by adipose tissue is stimulated by insulin (an indicator of glucose rich food intake) and glucocorticoids (Leroy, Dessolin et al. 1996; Slieker, Sloop et al. 1996; Zheng, Jones et al. 1996). Glucagon (an indicator of depleting glycogen store) and catecholamines are the counter-regulatory hormones known to inhibit leptin production (Ashwell, Czerwinski et al. 1999). An increase in food intake results in an enlargement of the adipose tissue mass. The enlarged adipose tissue cells when stimulated by insulin secrete higher amounts of leptin resulting in an increase in circulating leptin levels. In contrast, a decrease in food intake during
starvation results in a decrease in circulating leptin levels. Leptin present in the blood plasma exerts effects at various sites, including the hypothalamus, cerebellum, kidney, and lymph nodes (Margetic, Gazzola et al. 2002; Myers 2004). Overall the physiological response to a decrease in leptin involves an increase in appetite, enhancement of immune system activity, and a reduction in all energy-expending activities which are not important for immediate survival needs, for instance reproduction (Myers 2004).

**Leptin and Central Nervous System (CNS)**

Leptin null ob/ob mice and high-fat diet-induced obese mice respond to leptin treatment by decreasing food intake, and increasing activity; thus losing excessive body weight (Campfield, Smith et al. 1995). This effect of leptin is observed not only by intraperitoneal (i.p) but also intracerebroventricular (i.c.v) injection of leptin (Campfield, Smith et al. 1995). Since an i.c.v injection of leptin is sufficient to produce its effects on an animal’s feeding behavior and metabolism, this suggests that leptin acts on specific brain regions to produce those effects. Microinjection of leptin in extremely small doses into the hypothalamus enhances the effects of leptin on feeding (Jacob, Dziura et al. 1997). Leptin immunoreactivity is also found in various hypothalamic nuclei and the hippocampus, indicating that leptin, produced either in the peripheral organs or in the brain, reaches various brain regions and can thus affect brain functioning (Ur, Wilkinson et al. 2002). Besides feeding, leptin is involved in controlling other brain functions as well. It can act both as a cognitive enhancer and an antidepressant in adult rats (Harvey, Shanley et al. 2005; Lu, Kim et al. 2006). Various hippocampal functions such spatial memory formation, long-
term potentiation (LTP) and long-term depression (LTD) are also regulated by leptin (for more details see section on ‘Leptin and Hippocampal functioning’) (Li, Aou et al. 2002; Gerges, Aleisa et al. 2003; Wayner, Armstrong et al. 2004; Solovyova, Moult et al. 2009).

**Leptin Transport to Central Nervous System (CNS)**

So far, two distinct sources of leptin in the CNS have been discovered. One is the active transport of leptin across the blood-brain-barrier and the other is the local synthesis of leptin in the CNS. Although the main source of leptin in the body is the adipose tissue, many other potential sources (certain brain regions and other organs) have also been identified. In the CNS, leptin messenger RNA (mRNA) transcripts were detected in the cerebral cortex, cerebellum, pineal gland, hypothalamus and pituitary suggesting leptin synthesis in these regions (Morash, Li et al. 1999). The exact mechanism of local synthesis and release of leptin is still unknown. Although longer than a peptide (generally 50 amino acids or less), leptin (167 amino acids) might utilize a similar release machinery as some neuropeptides like oxytocin and vasopressin which are released from neuronal dendrites to affect neuronal functioning (Ludwig and Pittman 2003).

Although leptin can be locally synthesized in certain brain regions, the largest source of leptin circulating in the cerebrospinal fluid (CSF) is active transport across the blood-brain-barrier. Banks *et al.* suggested that leptin uses a saturable transport system to enter the brain. Iodinated-leptin injected i.p was detected in the brain, and the transport rate was higher than that for other peripheral cytokines (Banks, Kastin et al. 1996). This transport was inhibited by the presence of un-
iodinated leptin but not insulin, thus implying that the system is saturable and not regulated by insulin (Banks, Kastin et al. 1996). Banks et al. also showed that iodinated leptin present at normal concentration in the plasma reaches all brain regions (Banks, Clever et al. 2000). Using autoradiography, iodinated leptin injected i.p was detected in the choroid plexus, the hypothalamus, and the median eminence of the pituitary (Banks, Kastin et al. 1996). The amount of leptin reaching different brain regions and the saturation rate of these regions differ significantly (Banks, Clever et al. 2000). However, it is important to note that the leptin concentration observed in the hypothalamus was the same as in the hippocampus, thus indicating the hippocampus as another important site for leptin’s effects in the brain besides the hypothalamus (Banks, Clever et al. 2000).

**Leptin Receptors**

The leptin receptor (LepR, alternate abbreviation ObR) was cloned and expressed for the first time using expression cloning techniques from mouse choroid plexus (Tartaglia, Dembski et al. 1995). LepR is expressed from the db gene present on chromosome 4 (Tartaglia, Dembski et al. 1995; Chen, Charlat et al. 1996). It shares sequence and structural homology with the class 1 cytokine superfamily of receptors. It contains a number of fibronectin III domains and four cysteine residues on the extracellular domain, which are characteristic motifs of the class 1 cytokine superfamily (Tartaglia, Dembski et al. 1995; White and Tartaglia 1996). It exists as a homodimer that undergoes a conformational change after binding leptin (Devos, Guisez et al. 1997). This conformational change is thought to
cause its activation and initiation of number of downstream cascades discussed in detail in the section ‘LepR Signaling’.

**LepR Expression**

LepRs are widely expressed in the brain. LepR mRNA expression and immunoreactivity is observed in the hypothalamus, the hippocampus, cortex, choroid plexus, brain stem, cerebellum, amygdala, substantia nigra, and leptomeninges (Huang, Koutcherov et al. 1996; Mercer, Hoggard et al. 1996; Elmquist, Bjorbaek et al. 1998; Hakansson, Brown et al. 1998; Grill, Schwartz et al. 2002; Figlewicz, Evans et al. 2003). In the hippocampus, lepR immunoreactivity is localized in the CA1/CA3 and the dentate gyrus. In vitro studies show lepR immunoreactivity in the axon and the somato-dendritic region, especially in the synapses, of pyramidal neurons (Shanley, O'Malley et al. 2002). The presence of lepRs in the hippocampal synapses could allow leptin to regulate brain plasticity at the synaptic level.

**LepR Isoforms**

The *db* gene is alternatively spliced to express six different isoforms: LepRa to LepRf (Lee, Proenca et al. 1996). Each isoform has an extracellular N-terminal domain composed of 800 amino acids, and an intracellular C-terminal domain that is different in all isoforms. According to the length of the intracellular domain, the isoforms are categorized into three groups: the long form of the receptor (LepRb), the short forms of the receptor (LepRa, LepRc, LepRd, LepRf) and the secreted form of the receptor (LepRe).
The long form of lepR (LepRb) consists of a 300 amino acid long intracellular domain containing various motifs involved in the signal transduction. This receptor isoform has high expression in the hypothalamus (Mercer, Hoggard et al. 1996; Mercer 1996) and a specific loss of function mutation in it causes the obese phenotype of the lepRb mutant db/db mice (Lee, Proenca et al. 1996).

The short form lepRs contain a 30-40 amino acid long intracellular domain. As compared to the long form of lepRs, the short forms are abundantly expressed in the choroid plexus and the microvessels of the brain (Golden, Maccagnan et al. 1997; Bjorbaek, Elmquist et al. 1998). Also, the hypothalamus, which expresses the lepRb, lacks the short forms of the receptor (Bjorbaek, Elmquist et al. 1998). The presence of these short form receptors in the brain microvessels and the endothelium of other components of the brain circulatory network indicate that these receptors could be involved in leptin transport across the blood-brain-barrier (BBB). A strong correlation between plasma and CSF leptin levels is observed in humans. Leptin levels in the CSF increase with increasing adiposity, following the trend observed in the plasma (Schwartz, Peskind et al. 1996). Mice lacking all lepRs show a decreased uptake of leptin into the brain. High-fat diet-induced obese mice also have a reduction in leptin uptake into the brain (Hileman, Pierroz et al. 2002). The leptin transport system is saturable, evident from the fact that as obesity progresses in mice the rate of leptin transport across the BBB decreases (Banks and Farrell 2003). On the other hand, with starvation, there is an increase in triglycerides in the blood that also result in reduction of leptin transport across the BBB (Banks, Coon et al. 2004). These studies together confirm the presence of a saturable leptin transport system that utilizes the short form lepRs. The lepRa and
leprc isoforms are especially abundant in the choroid plexus and microvessels (Bjorbaek, Elmquist et al. 1998; Hileman, Pierroz et al. 2002) and therefore, are the most likely candidates for leptin transporters across the BBB.

**LepR Signaling**

The leprs belong to the class1 superfamily of cytokine receptors (Tartaglia, Dembski et al. 1995). They exist in dimer form, and this dimerization is required but not sufficient for receptor activation (Devos, Guisez et al. 1997). Leptin binding to the receptors causes heterodimerization of the various lepr isoforms (White and Tartaglia 1999); but unlike in the case of other cytokines, leprs do not bind to other cytokine receptors (Nakashima, Narazaki et al. 1997; Nakashima, Narazaki et al. 1997). Heterodimerized leprs recruit two Janus Kinases (JaK) that trans-phosphorylate to activate each other. Trans-phosphorylated JakS phosphorylate of multiple tyrosine residues present on the intracellular domain of the receptor (Ghilardi and Skoda 1997). Various downstream targets can bind to these phospho-tyrosine residues to initiate different signaling cascades. Most short forms of the leprs do not contain any of these tyrosine residues and therefore do not activate most of the signaling cascades found downstream of lepb (the long form) (Bjorbaek, Uotani et al. 1997). LepRa (a short form) has been shown to activate the mitogen-activated protein kinase (MAPK) pathway in certain cell types (HEK (Bjorbaek, Uotani et al. 1997), CHO (Yamashita, Murakami et al. 1998)). This can occur even in the absence of the phospho-tyrosine residues because lepRa retains the ability to recruit and activate Jak2, which can further activate the MAPK pathway (Bjorbaek, Uotani et al. 1997).
Signal Transducers and Activators of Transcription (STAT) signaling: STATs are a family of transcription factors that mediate leptin’s effects on feeding in the hypothalamus. Only lepRb contain the phosphor-tyrosine residue that binds to STAT, and is therefore the only lepR isoform to activate the STAT signaling. It activates STAT 3, 5 and 6 but not STAT 1, 2 and 4 (Ghilardi, Ziegler et al. 1996). STAT3 binds to phospho-tyrosine residue 1138 and STAT5 binds to phospho-tyrosine residue 1107 (Hekerman, Zeidler et al. 2005). STAT binding to lepRb leads to STAT phosphorylation, dimerization and re-localization to the nucleus (Fruhbeck 2006). Long term STAT3 activation results in over-expression of suppressor of cytokine signaling 3 (SOCS3), a feedback inhibitor of leptin signaling (Bjorbaek, Buchholz et al. 2001). SOCS3 binds Jak2 and inhibits both the PI3K and STAT signaling cascades.

MAPK signaling: Both lepRa and lepRb activate the MAPK signaling cascade. The activation by lepRa is below maximal (Bjorbaek, Uotani et al. 1997) because it lacks the phosphor-tyrosine residue 985 required for complete activation of the MAPK pathway (Banks, Clever et al. 2000). The downstream targets of this pathway vary between different cell types.

PI3K signaling: This signaling pathway integrates the leptin and insulin signaling cascades. Insulin signaling recruits and activates insulin regulatory substances (IRS) that can interact with other signaling cascades. LepRs bind IRS1/2, which further lead to activation of the PI3K (Fruhbeck 2006).
Leptin and Brain development

Numerous studies in different mammalian species have identified the expression pattern of leptin and lepRs during developmental phases of the brain. Leptin and lepR expression was first detected in the leptomeninges and choroid plexus of embryonic day 14 (E14) mice brain using western blot and immunocytochemistry (Hoggard, Mercer et al. 1997). Using RT-PCR, lepRb mRNA was detected in the brain of E10 mice (Udagawa, Hatta et al. 2000). The earliest detection of lepR immunoreactivity in rats is at E14 in the ventricular layer, which
contains premature neuronal cells at this stage of brain development (Matsuda, Yokota et al. 1999).

The expression pattern of lepR changes across brain regions throughout development, suggesting its role in development of different brain regions. Using in-situ hybridization, lepRb expression was located in the ventricular layers of several brain regions of E11 to E18 mice fetuses (Udagawa, Hatta et al. 2000). By E18, lepRb expression was detected in the newly formed hypothalamic nuclei (arcuate nucleus and ventro-medial nucleus) of mice (Udagawa, Hatta et al. 2000). In rats, lepRb immunoreactivity can be detected in the paraventricular nucleus of the hypothalamus at E18 (Matsuda, Yokota et al. 1999). This immunoreactivity increases and is detected at higher levels in postnatal day 0 (P0) in rats, however these levels are still lower than adult levels (Matsuda, Yokota et al. 1999). Using in-situ hybridization and a leptin binding assay, lepRb expression was found to be present in the arcuate and ventral hypothalamic nuclei of E21 rats (Carlo, Meyerhof et al. 2007). LepR expression can be localized in the thalamus and the hippocampus of P3 rats (Carlo, Meyerhof et al. 2007).

In addition to lepR expression, leptin expression also varies in different brain regions throughout development. Local leptin expression could indicate a role for specific brain regions in providing leptin to the brain during development when adipose deposits are minimal. High leptin mRNA levels are observed in the pituitary from P7 to P14 in rats and this drops significantly at P22 (Morash, Wilkinson et al. 2001). On the other hand, leptin mRNA levels remain constant in the hypothalamus from P2 to adulthood in rats (Morash, Wilkinson et al. 2001). Studies have shown that leptin in neonates does not have the same physiological effects as it does in
adults. Leptin reduces food intake and body weight in adults, but acute and chronic leptin treatment (3mg/kg body weight i.p injection) fails to alter food intake or body weight of P10 rats (Proulx, Richard et al. 2002). Leptin protein and mRNA levels surge from P4 to P16 in mice without affecting their body weight during this developmental period (Ahima, Prabakaran et al. 1998). Unlike in adult mice, food deprivation does not decrease leptin levels in P8 mice (Ahima, Prabakaran et al. 1998). This complete uncoupling of leptin and feeding behavior during early postnatal development cannot be ascribed to lack of leptin signaling in neonates because acute leptin treatment (3mg/kg body weight i.p injection) results in an increase of SOCS3 expression in the arcuate nucleus of the hypothalamus in P10 rats (Proulx, Richard et al. 2002) suggesting activation of leptin signaling. In the hippocampus, acute leptin treatment increases activated/phosphorylated-Erk1/2 immunoreactivity without increasing activated/phosphorylated-STAT3 immunoreactivity in the CA1 and dentate gyrus of P10 rats (Walker, Long et al. 2007) suggesting activation of leptin signaling but through a different pathway from what was observed in the hypothalamus. Since leptin signaling is intact in neonates, this indicates a different role for leptin during development then it has in adults. The following studies strongly support the role for leptin in brain development. Leptin null ob/ob mice have lower brain weight and cortical volume (Steppan and Swick 1999). A two week long leptin treatment of four week old ob/ob mice increases their brain weight and brain DNA (Steppan and Swick 1999). Leptin null ob/ob mice and lepR mutant db/db mice have an immature pattern of expression of synaptic (syntaxin-1, synaptobrevin) and glial proteins (GFAP) and decreased myelination (Ahima, Bjorbaek et al. 1999). Bouret et al. provided
convincing evidence of involvement of leptin in hypothalamic development (Bouret, Draper et al. 2004). They found that leptin null ob/ob mice have lower fiber density in the paraventricular nucleus of the hypothalamus during neonatal phase (P10, P16) as well as adulthood (P60) as compared to the wild type controls. This deficiency was partially restored to normal levels by treating the ob/ob mice with leptin during the early developmental period (P0-P14).

The Hippocampus

Psychologists and neurophysiologists have researched hippocampal functions and behaviors for several decades now (Rosenzweig and Leiman 1968; Sprick 1995; Roxo, Franceschini et al. 2011). Scoville and Milner’s groundbreaking work describing the famous amnesiac HM who suffered bilateral hippocampal lesions placed the hippocampus at the epicenter of learning and memory research (Scoville and Milner 1957). Ever since, the hippocampus has been shown to be essential for formation of various types of memories (working, declarative, spatial, emotion-related, food-related) (O’Keefe and Dostrovsky 1971; Eichenbaum, Schoenbaum et al. 1996; Eichenbaum 2000; Kesner 2000; Burgess, Maguire et al. 2002; Benoit, Davis et al. 2010). It is also part of the limbic system and is therefore important for emotional and motivational regulation (Isaacson and Wickelgren 1962; Flynn 1969; Isaacson 1984).

Leptin and Hippocampal Functioning

The extra-hypothalamic expression of lepRs in brain regions not involved in regulating feeding behavior suggests that there are diverse functions of this
cytokine. A variety of recent studies have implicated leptin as a modulator of hippocampal functioning. The hippocampus is important for memory formation and consolidation. Mice subjected to T-maze footshock avoidance tests have better retention of the fear memory if treated with intra-hippocampal leptin infusion immediately after the test (Farr, Banks et al. 2006). Senescence accelerated mice 8 (SAMP8) mice, which suffer from memory deficits as they age, show improved memory retention at 4 and 12 months of age after chronic leptin treatment (Farr, Banks et al. 2006). This effect of leptin could be dose dependent since rats treated with intravenous injections of low doses (5-50µg/kg body weight) of leptin have better retention of fear memory (in passive avoidance test) and spatial memory (water maze test) as compared to rats treated with high doses (500µg/kg body weight) of leptin (Oomura, Hori et al. 2006). Besides regulating memory formation, leptin also affects hippocampus-dependent emotions. Rats subjected to chronic unpredictable stress model for depression have reduced leptin in their plasma (Lu, Kim et al. 2006). When treated with intra-hippocampal leptin infusion, these rats are relieved of their depressive symptoms (Lu, Kim et al. 2006).

Leptin also has neuroprotective effects on the hippocampus. It increases neuronal survival rates in hippocampal neuronal cultures treated with cytotoxic agents via a STAT3 and PI3K (not MAPK) dependent pathway (Guo, Jiang et al. 2008). The neuroprotective effects of leptin are also demonstrated in in vivo studies. Intracerebroventricular injection of leptin protects rats against seizure damage and ischemic injury via a STAT3 dependent pathway (Guo, Jiang et al. 2008; Zhang and Chen 2008).
The hippocampus is one of the few brain regions where neurogenesis has been observed in the adult brain. *In vitro* studies have revealed that leptin increases cell proliferation at low concentrations (1-3nM) but not at high concentrations (10-30nM) (Garza, Guo et al. 2008). Also, chronic leptin treatment in adult mice increases cell proliferation in the dentate gyrus without affecting differentiation and survival of the newly-proliferated cells via a STAT3 but not MAPK dependent pathway (Garza, Guo et al. 2008).

One of the many ways by which leptin can regulate hippocampal functioning is by modifying synaptic plasticity. LepR deficient zucker rats and *db/db* mice have impaired spatial memory and hippocampal slices prepared from these animals have impaired synaptic long-term potentiation (LTP) and long-term depression (LTD) in the CA1 region (Li 2002). In 2001, Shanley and coworkers were the first to show that *in vitro* leptin can alter hippocampal synaptic strength by converting short-term potentiation (STP) to LTP (Shanley, Irving et al. 2001). This conversion occurred due to facilitation of N-methyl-D-aspartate receptor (NMDAR) function by leptin via a PI3K and MAPK dependent pathway (Shanley, Irving et al. 2001).

Interestingly, *in vivo* leptin effects LTP in a dose dependent manner. Wayner et al. reported an enhancement of LTP *in vivo* at 1µM concentration of leptin but an inhibition of LTP at all lower and higher doses of leptin (Wayner, Armstrong et al. 2004). Leptin can also reverse LTP at high concentrations when applied within 30 minutes of LTP induction (Moult, Milojkovic et al. 2009). The LTD inducing effects and depotentiating effects (LTP reversal) of leptin may be part of the homeostatic mechanisms involved in maintaining the excitability of neurons and preventing excitatory saturation of potentiated synapses.
Leptin can also regulate hippocampal function by impacting neuronal morphology. Leptin is involved in rapid actin cytoskeleton rearrangement via the PI3K pathway (O'Malley, MacDonald et al. 2007). In hippocampal neuronal cultures, leptin stimulates dendritic filopodia formation and motility within 30 minutes (O'Malley, MacDonald et al. 2007). This cytoskeletal rearrangement is activity dependent since it can be blocked by TTX (a Na+ channel blocker), AP-5 (NMDAR inhibitor). Leptin stimulation also increased the number of synapsin-positive puncta in this study, indicating that the filopodia matured to functional synapses.

The various effects of leptin on hippocampal functioning are observed not only during adulthood but also during the neonatal period. Chronic leptin treatment from P2-10 in rat pups increases the expression of NR1 subunit of the NMDAR while decreasing the expression of NR2B subunit of NMDAR at P10 (Walker, Long et al. 2007). These changes in NMDAR subunit expression are long lasting and can be detected at P70 in adult rats (Walker, Long et al. 2007). Chronic leptin treatment in neonatal rats (P2-10) also modifies the expression of the important synaptic proteins synapsin and synaptophysin but these changes do not persist into adulthood (Walker, Long et al. 2007). Nevertheless, this same leptin treatment did result in increased expression of another synaptic protein called SNAP-25 in adult animals (P70) (Walker, Long et al. 2007).

**Synapse formation**

The dendritic arbor of a neuron determines the amount and variety of signals a neuron can receive and integrate. It passes through 4 phases of development; initiation, outgrowth, branching, and synapse formation (Scott and Luo 2001). A
spine is an actin-rich short protrusion from the dendrite that is the primary site of excitatory synapses on a neuron (Shepherd 1996). It matures as it receives pre-synaptic input and forms a functional synapse (Nimchinsky, Sabatini et al. 2002). Dendritic spines are classified into three different categories depending on the size of the post-synaptic density (PSD) in its head/tip and the size of its neck; namely filopodia, stubby and mushroom (Harris and Kater 1994). The type of spine may represent its state of maturity or synaptic efficacy and has the ability to influence the overall input/output of a neuron. Neurotrophic factors, such as nerve growth factor, neurotropin 3, neurotropin 4/5 and brain derived neurotrophic factor have been shown to regulate dendritic growth and eventual synapse formation (Horch, Kruttgen et al. 1999; Scott and Luo 2001; Chapleau, Larimore et al. 2009). Besides neurotrophic factors, activity level of a neuron also affects the growth and maturation of its dendritic arbor. Inhibition of NMDARs results in the collapse of the dendritic arbor and therefore decreases total synapse number (Wayman, Davare et al. 2008). This activity dependent regulation of dendritic development is thought to be important for learning and memory induced synaptic plasticity. CyclicAMP response element-binding protein (CREB), an activity dependent transcription factor, has been shown to regulate the expression of cytoskeletal proteins that mediate dendritic development (Redmond, Kashani et al. 2002; Wayman, Impey et al. 2006).

Considering that dendritic development is important for optimal neuronal functioning, it is not surprising that numerous developmental disorders, such as autism, fragile-X syndrome, fetal alcohol syndrome and down’s syndrome have
been correlated with abnormal dendritic growth and spine formation (Irwin, Galvez et al. 2000; Dierssen and Ramakers 2006) (Nimchinsky, Sabatini et al. 2002).
References


CHAPTER 1

Leptin-activated TrpC current in the hippocampus is important for spine formation.

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Abstract

Leptin acts on hippocampal pyramidal neurons to promote actin reorganization and filopodia formation as a first step in spine and glutamatergic excitatory synapse development. In this report, we describe a leptin-induced slow-activating current that is required for filopodia formation in cultured hippocampal neurons. This current was blocked by targeted knockdown of the leptin receptor as well as the TrpC channel inhibitors SKF96365 and 2-APB. Targeted knock-down of TrpC1 and 3, but not TrpC5, channels also eliminated the leptin current. Leptin induces the TrpC current by trafficking TrpC1 subunit containing channels to the membrane through the activation of CaMKK\textbeta, CaMKI\gamma and \textbeta-Pix to stimulate Rac1. Furthermore, the components of pathway that required for leptin-induced TrpC trafficking and activation are also required for leptin-induced filopodia formation.
thus elucidating a critical pathway underlying leptin’s induction of dendritic morphological changes that initiate spine and excitatory synapse formation.

Introduction

The hormone leptin is a key regulator of energy homeostasis in adults and its effects on food intake and energy regulation have been extensively studied (Ahima and Flier 2000; Farooqi and O’Rahilly 2009; Dieguez, Vazquez et al. 2011; Gautron and Elmquist 2011). Interestingly however, leptin does not alter food intake or body weight during neonatal development, even though leptin levels surge after birth from postnatal day 7-14 (Ahima, Prabakaran et al. 1998). This post-natal surge is essential for the appropriate development of neuronal connections in the hypothalamus, consistent with leptin having neurotrophic actions (Bouret, Draper et al. 2004). Furthermore, leptin also stimulates synapse formation in the mature hypothalamus suggesting it also acts as a neurotrophic factor in adults (Pinto, Roseberry et al. 2004).

Leptin receptors (LepR) are expressed widely throughout the brain, including the hippocampus (Huang, Koutcherov et al. 1996; Mercer, Hoggard et al. 1996). Deficiency in either leptin or LepR signaling during development leads to impaired hippocampal function and associated aberrations in hippocampal behaviors such as depression, anxiety and decreased memory (Li, Aou et al. 2002; Sharma, Elased et al. 2010; Yamada, Katsuura et al. 2011). LepR deficient mice (db/db) have lowered spine density in the dentate gyrus, CA1 and CA3 of the hippocampus compared to their wildtype counterparts (Stranahan, Lee et al. 2009) (Figure1, chapter2). Furthermore, we recently showed that leptin stimulates the development and
maturation of hippocampal spines through activation of CREB-dependent transcription (Figure 4 and 5, chapter 2). However, while leptin has also been shown to stimulate filopodia formation, the first stage to forming a spine (O’Malley, MacDonald et al. 2007), the molecular mechanisms by which leptin initiates this critical process are unknown.

Brain derived neurotrophic factor (BDNF) is a potent stimulator of spine formation in the hippocampus (Chapleau, Larimore et al. 2009). Recently, TrpC channels have been shown to be important for BDNF-induced spine formation in the hippocampus (Amaral and Pozzo-Miller 2007; Amaral and Pozzo-Miller 2007; Li, Calfa et al. 2010) and leptin has also been shown to activate TrpC current in the arcuate and premammillary nucleus of the hypothalamus (Qiu, Fang et al. 2010; Williams, Sohn et al. 2011). Therefore, in this study we established whether leptin activates a TrpC current in developing hippocampal neurons and whether this current is required for leptin-induced spine formation. As TrpC channels are rapidly inserted into the membrane by other growth factors (Bezzerides, Ramsey et al. 2004; Amaral and Pozzo-Miller 2007), we also determined whether leptin stimulated trafficking of TrpC channels. Lastly, we determined whether Rac1 was required and whether it involved a novel signal transduction pathway for leptin, the calcium/calmodulin dependent kinase (CaMK) cascade, as this cascade has recently been shown to be an important regulator of the actin cytoskeleton and synapse formation (Nakayama, Harms et al. 2000; Saneyoshi, Wayman et al. 2008; Wayman, Lee et al. 2008).
**Results**

*Acute leptin treatment induces protrusion formation as well as an inward current in hippocampal neurons*

Dendritic protrusions such as filopodia or thin spines are potential sites of mature excitatory synaptic connections on a neuron. Various factors can create permissive conditions that prompt a neuron into developing more synaptic contacts with its neighboring neurons. Similar to previous reports, we observed a rapid increase in dendritic protrusions in day in vitro 8 (DIV8) hippocampal neurons after 30 minutes of leptin stimulation (Figure1A and B) (O’Malley, MacDonald et al. 2007). This effect was mediated by direct binding of leptin with the long isoform of its receptor (lepRb) since neurons transfected with a targeted shRNA to lepRb did not show any increase in protrusion density (Figure1A and B). Leptin also stimulated a slow activating cationic inward current ($I_{\text{leptin}}$) of approximately 30.54 ±1.511 pA in DIV8-9 hippocampal cultured neurons (Figure1C and D). This inward current is barely observed at low leptin concentration (0.93pA and 1.52pA at 1nM and 3nM respectively) but a significant increase is observed at 10nM (7.85pA) which increases further at 30nM (18.7pA) (Figure1C). The amplitude of this leptin-activated current does not tend to increase any further with increasing leptin concentration. Leptin requires the expression of lepRb for activating this current as well since reducing lepRb expression by shRNA completely blocks this current (Figure1D and E).
Figure 1: Leptin induces protrusion formation as well as an inward current in hippocampal neurons. (A–B) Cultured hippocampal neurons were transfected with mRFP-βactin ±shLepR on DIV6 and treated with leptin on DIV8 for 30min. A, Representative images and B, Average protrusion density under specified conditions are shown. (C) Indicated concentration of leptin was bath applied onto DIV8-9 hippocampal neurons and the resulting current was recorded. Average peak amplitude at specified concentrations is shown. (D–E) Cultured hippocampal neurons were transfected with mRFP-βaction ±shLepR on DIV6 and leptin current was recorded on DIV8-9. D, Representative traces and E, average peak amplitude of leptin current under control and shLepR conditions is shown. Protrusion and electrophysiology data was analysed using ANOVA followed by Tukey’s post-hoc analysis (±SEM, ***p<0.001 compared to control).

Pharmacological characterization of leptin-induced current

As leptin activates TrpC-mediated current in POMC neurons of the arcuate nucleus (Qiu, Fang et al. 2010), we next determined if this slow-activating current was mediated by TrpC channels. Pre-treatment with SKF96365 (SKF), a commonly
used TrpC channel blocker (Clapham 2005), attenuated both leptin’s effects on protrusion formation (Figure 2A and B) and the leptin-induced current (Figure 2C and D). Extracellular application of another TrpC blocker, 2-APB, (Clapham, Runnels et al. 2001), also attenuated $I_{\text{leptin}}$ further suggesting that the leptin-activated current required functional TrpC channels (Figure 2D).

**Figure 2: Leptin-induced protrusion formation and current are blocked by SKF. (A-B)** Cultured hippocampal neurons were transfected with mRFP-βactin on DIV6 and treated with leptin on DIV8 for 30min. SKF treatment was applied 20minutes prior to leptin treatment. **A,** Representative images and **B,** Average protrusion density under specified conditions are shown. **(C-D)** Leptin was bath applied to DIV8-9 cultured hippocampal neurons and leptin current was recorded. SKF was bath applied prior to leptin application and was washed out as indicated **D,** Representative traces and **E,** average peak amplitude of leptin current under control, SKF and 2-APB conditions is shown. Protrusion and electrophysiology data was analysed using ANOVA followed by Tukey’s post-hoc analysis ($\pm$SEM, $$$p<0.001$ compared to control, $###p<0.001$ compared to leptin).
Leptin current requires TrpC 1 and 3 but not 5 expressions

The TrpC channel family consists of 7 different subunits that can form either homomeric or heteromeric channels (Clapham 2005). The presence of specific subunits in a TrpC heteromer determines its pharmacological properties and ionic permeability. At millimolar concentrations, lanthanum is a general blocker of TrpC channels but at micromolar concentrations it potentiates TrpC4 or 5 channel currents (Schaefer, Plant et al. 2000; Strubing, Krapivinsky et al. 2001). In contrast to the leptin-current observed in the arcuate nucleus (Qiu, Fang et al. 2010), 100µm La^{3+} did not potentiate I_{leptin} in hippocampal neurons. 100 uM lanthanum partial reduced and 1 mM Lanthanum completely blocked I_{leptin} (control, 20.47±1.10 pA; 100µM La^{3+}, 12.8±3.35 pA; 1mM La^{3+}, 0.57±1.07 pA) (Figure3A), indicating that the TrpC channel mediating I_{leptin} does not contain TrpC4 and 5 subunits.

TrpC channels are generally considered non-selective cationic channel that are permeable to both Ca^{2+} as well as Na^{+} with a subunit-dependent preference (Clapham, Runnels et al. 2001). Reducing the calcium concentration from 3mM to 0.5mM in the bath had no significant effect on the amplitude of I_{leptin}, while reducing the sodium concentration from 140mM to 5mM strongly reduced the amplitude of the leptin-induced current (Figure3A). Furthermore, we found I_{leptin} was partially reduced by increasing extracellular magnesium (control, 21.27±1.62 pA; 4mM Mg^{2+}, 6.65±2.03 pA) (Figure3A). These two properties are again consistent with a TrpC1/3/6 type of channel.

In order to confirm the molecular composition of the TrpC channel involved we used targeted shRNAs or siRNAs to reduce expression of various TrpC subunits and determine their effect(s) on the leptin-induced current. Expression of TrpC1
and TrpC3 was found to be essential for the leptin-induced current as targeted knockdown of either subunit blocked $I_{\text{leptin}}$ (Figure 3B and C). However, consistent with our pharmacological data, TrpC5 expression was not required as shRNAs targeting TrpC5 had no effect (Figure 3B and C).

**Figure 3: Leptin current is mediated by TrpC1 and 3 channel.** (A) DIV8-9 cultured hippocampal neurons were treated as specified and leptin current was recorded. Amplitude of leptin current under indicated conditions is shown. (B-C) Cultured hippocampal neurons were transfected with mRFP-βactin ± shTrpC1, siTrpC3, shTrpC5 or scrambled shRNA on DIV6. Leptin was bath applied on DIV8-9 neurons and leptin current was recorded. **B**, Representative traces and **C**, average peak amplitude of leptin current under indicated conditions is shown. Data was analysed using ANOVA followed by Tukey’s post-hoc analysis (±SEM, **p<0.01, ***p<0.001 compared to control).

**Leptin initiates the CaMK pathway to activate TrpC current**

We next determined the molecular pathway induced by leptin that activates TrpC1/3 channels. Leptin increased CaMKI phosphorylation/activation in DIV8 cultured hippocampal neurons within 5 minutes, an effect that lasted for at least 10
minutes (Figure 4A and B). Consistent with this pathway being required for activation of $I_{\text{leptin}}$, pretreatment with STO-609, a CaMKK inhibitor, completely attenuated leptin’s induction of the current (Figure 4C). Targeted knockdown of specific isoforms of CaMKK also attenuated $I_{\text{leptin}}$, with shCaMKKβ completely blocking $I_{\text{leptin}}$, while shCaMKKα partially reduced the leptin current (Figure 4C). We next determined that activation of CaMKIγ was required for $I_{\text{leptin}}$ as shCaMKIγ, but not shCaMKIβ, completely attenuated the current as well (Figure 4C).

![Figure 4: Leptin-activated CaMK pathway is required for the leptin current. (A-B)](image)

DIV8 cultured hippocampal neurons were treated with leptin for various durations (5-30 minutes) and then lysed using RIPA. Samples were run on a SDS-PAGE and blotted with anti-pCaMKI and anti-Erk1/2 antibody. **A**, Representative western blot and **B**, average pCaMKI band intensity normalized to Erk1/2 (loading control) under indicated conditions is shown. **C** Cultured hippocampal neurons were transfected with mRFP-βactin ± shCaMKKα, shCaMKKβ, shCaMKIγ or shCaMKIβ on DIV6. Leptin was bath applied on DIV8-9 neurons and leptin current was recorded. STO-609 was bath applied prior to leptin application. Average peak amplitude of leptin current under indicated conditions is shown. Western blot data was analyzed using Student’s t-test. Electrophysiological data was analysed using ANOVA followed by Tukey’s post-hoc analysis (±SEM, *p<0.05, **p<0.01, ***p<0.001 compared to control).
βPix activated Rac signaling is required for leptin-induced TrpC current

We have previously shown that CaMKK, CaMKI and βPix (a RacGEF) form a signaling complex in rat hippocampal neurons and in response to synaptic activity they activate Rac1 and initiate spine formation (Saneyoshi, Wayman et al. 2008). Therefore, we next determined if leptin increased the phosphorylation state of βPix and if this phosphorylation event is CaMKK/CaMKIγ dependent. To test this, hippocampal neurons were transfected with myc-tagged βPix alone or along with short-hairpin constructs targeting the CaMK pathway. βPix was then immunoprecipitated with an anti-myc antibody following leptin stimulation. Similar transfection and pulldown efficiency across samples was confirmed by comparing input samples as well as immunoprecipitated samples using an anti-myc antibody. Leptin treatment increases βPix phosphorylation 5-10 minutes post-treatment (Figure 5A and B). The leptin-induced phosphorylation of β-Pix was dependent on CaMKK/CaMKIγ as leptin had no effect in neurons pre-treated with STO-609 or transfected with shCaMKIγ (data not shown and Figure 5A and B). We next confirmed the requirement of β-Pix and Rac1 activity for I_{leptin}. Blocking β-Pix and Rac1 expression by shRNAs (shβ-Pix and shRac1) or activity by dominant negatives (dnRac1 for Rac1) blocked I_{leptin}, as did over-expression of DHm, a β-Pix mutant lacking the GEF domain or β-PixS516A, a mutant construct that cannot be phosphorylated by CaMK1 at the canonical site (Figure 5C).
**Figure 5: β-Pix phosphorylation and activation is required for leptin current. (A-B)** DIV5 cultured hippocampal neurons were transfected with β-Pix-6myc ±shCaMKIγ and stimulated with leptin for various durations on DIV8. Cells were lysed in RIPA and immunoprecipitated using anti-myc antibody. Samples were loaded on a SDS-PAGE and blotted with anti-phospho β-Pix antibody. A, Representative western blot and B, average relative phospho β-Pix band intensity of immunoprecipitated samples normalized to input samples for indicated conditions is shown. (C) DIV5 cultured hippocampal neurons were transfected with mRFP-βactin along with the specified constructs. Leptin was bath applied to DIV8-9 neurons and leptin current was recorded. Average peak amplitude of leptin current under indicated conditions is shown. Western blot data was analyzed using Student’s t-test.

Electrophysiological data was analysed using ANOVA followed by Tukey’s post-hoc analysis (±SEM, *p<0.05, **p<0.01, ***p<0.001 compared to control).
Leptin increases TrpC1 trafficking to the membrane via a CaMK/βPix-dependent pathway

The slow-initiation of leptin-activated TrpC current in our experiments could be attributed to a required trafficking event. To test this hypothesis, we conducted surface biotinylation experiments to detect changes in the amount of TrpC channels in the neuronal membrane before and after leptin stimulation. A TrpC1 construct tagged with a flag epitope was transfected in DIV5 hippocampal neurons and on DIV8 neurons were treated with 50 nM leptin. After leptin stimulation surface expressed TrpC1 was biotinylated. Using an anti-flag antibody TrpC1 was immunoprecipitated and concentrated from each sample. After accounting for transfection and pull-down efficiency, all samples were compared for membrane-bound TrpC1 levels using an avidin antibody that specifically binds biotinylated proteins. Leptin increased TrpC1 levels in the membrane 10 minute after leptin treatment and this increase was sustained even 20 minutes later (Figure 6A and B). Leptin didn’t alter total TrpC levels in the neuron in this time period as there was no difference in the input samples between conditions. Co-transfection of the TrpC1-flag construct with shCaMKIγ or βPix(S516A) didn’t change the initial levels of TrpC channel in the membrane compared to controls but they completely blocked leptin from stimulating TrpC trafficking (Figure 6A and B).
**Figure 6: Leptin increases TrpC1 trafficking to the membrane via a CamK/βPix-dependent pathway. (A-B)**

DIV5 cultured hippocampal neurons were transfected with TrpC1-flag ±shCaMKIγ or βPixS516A and treated with leptin on DIV8 for various durations. Cells were biotinylated, quenched and lysed, and immunoprecipitated using anti-flag antibody. **A**, Representative western blot and **B**, relative average intensity of avidin band normalized to flag band under indicated conditions is shown. Western blot data was analyzed using Student’s t-test (±SEM, *p*<0.05, **p**<0.01, ***p**<0.001 compared to control).

**TrpC channel trafficking and activity is required for leptin-induced spine initiation in hippocampal neurons**

Finally, we determined the requirement of the signaling cascade initiated by leptin for TrpC channel trafficking in leptin’s effects on protrusion formation. Inhibiting CaMKK activity using STO-609 or CaMKIγ expression using a shRNA blocks leptin’s effects on protrusion formation, indicating the importance of the CaMK (Figure 7B). Activation of the CaMK pathway and β-Pix phosphorylation and
subsequently Rac1 activation is also required as leptin also failed to induce filopdia in neurons transfected with β-PixS516A or shRac1 (Figure 7A and B). TrpC1 and 3 were also required for leptin-induced protrusion formation, consistent with them forming the required TrpC channel as targeted knockdown of either blocked leptin’s effects (Figure 7A and C). Together, these data confirm that the signaling cascade that we describe for leptin-induced TrpC trafficking is also required for leptin-induced protrusion formation and presumably spine initiation.

Figure 7: TrpC channel trafficking and activity is required for leptin-induced spine initiation in hippocampal neurons. (A-C) DIV5 cultured hippocampal neurons were transfected with mRFP-βactin along with various specified constructs. Neurons were stimulated with leptin for 30 minutes on DIV8 and then fixed immediately. A, Representative
images, **B and C**, average protrusion density under indicated conditions in shown. Data was analyzed using ANOVA followed by Tukey post-hoc analysis (±SEM, *p<0.05, **p<0.01, ***p<0.001).

**Discussion**

Leptin effects hippocampal neuronal functioning, synaptic plasticity and related behaviors (Harvey, Shanley et al. 2005; Harvey 2007). Leptin receptors are found in the CA1, CA3 and DG regions of the hippocampus (Mercer, Hoggard et al. 1996; Shanley, O'Malley et al. 2002; Guo, Lu et al. 2012). However, while leptin has been shown to induce synapse formation (Pinto, Roseberry et al. 2004)), the molecular mechanisms by which leptin initiates this process are not known. Here we show for the first time that leptin induces filopodia formation by activating a TrpC current mediated by the TrpC1 and TrpC3 subunit containing channels. Furthermore, we show this current is induced by the translocation of the channel to the plasma membrane following activation of β-Pix and Rac 1 downstream of the CaM KK/CaMK1γ cascade.

TrpC channels have previously been shown to also play a critical role in BDNF-dependent spine formation (Amaral and Pozzo-Miller 2007; Davare, Fortin et al. 2009; Li, Chen et al. 2012), suggesting that TrpC channels may be a common mechanism by which neurotrophic factors induce spine formation. We also show that leptin specifically induces a slow activating current that is mediated by TrpC1 and TrpC3, but not TrpC5, channels and that these channels are required for filopodia formation. TrpC3, but not TrpC5, channels are also required for BDNF induction of both a slow-activating current and spine formation in hippocampal
neurons (Amaral and Pozzo-Miller 2007), suggesting BDNF and leptin may utilize a common pathway.

The predicted involvement of TrpC1 channel in leptin’s action however is novel. The neurotrophic factors BDNF and NGF have been shown to traffic TrpC3 and TrpC5 channels to the membrane (Bezzerides, Ramsey et al. 2004; Amaral and Pozzo-Miller 2007). This is the first demonstration of TrpC1 being trafficked following treatment with a neurotrophic factor. The TrpC1 channel is primarily proposed to mediate calcium-activated calcium release (Liu, Singh et al. 2003; Ambudkar 2007). This is also the first demonstration of a role for this channel in spine formation.

Interestingly, leptin has previously been shown to activate a TrpC4 or 5 channel in the hypothalamus (Qiu, Fang et al. 2010; Williams, Sohn et al. 2011) suggesting leptin can activate different TrpC currents in different brain regions. Our data suggests the leptin does not activate TrpC4 or 5 in hippocampal neurons. One consequence of activating different types of TrpC channels is the different kinetic and regulatory properties that are conferred by the diverse subunit composition.

The long isoform of the leptin receptor (LepRb) is a cytokine receptor that can initiate multiple signaling cascades; such as the MAPK, PI3K and PLC pathway (Myers 2004). Here we show for the first time that leptin increases phosphorylation of CaMKI and that this activation was required for activation of the leptin current as well as leptin-induced spine formation. We also show that both CaMKKβ and CaMKIγ are required. Both CaMKI and CaMKK require Ca²⁺/CaM binding in addition to phosphorylation for activation (Wayman, Lee et al. 2008). The source of calcium is as yet unknown; Amaral et. al. reported that BDNF-induced translocation of
TrpC3 in hippocampal neurons requires release of intracellular calcium stores via the IP3 receptors (Amaral and Pozzo-Miller 2007) and requires PLC. Qiu et. al. also showed that leptin-induction of a TrpC current in the hypothalamus requires PLC, although they concluded that IP3 receptors were not involved (Qiu, Fang et al. 2010). We also found a requirement for PLC, however we did not address the requirement of IP3. Calcium could also enter through calcium channels, other TrpC channels, or even NMDA receptors as NMDA receptor activity is vital for leptin’s effects on filopodia formation (O’Malley, MacDonald et al. 2007).

Leptin-activation of the CaMKK/CaMKI cascade leads to activation of β-Pix, a Rac1 GEF, and consequently Rac1 activation. We had previously reported that CaMKK, CaMKI, β-Pix and Rac1 exist in a complex that can respond to synaptic activity to promote the rearrangement of the actin cytoskeleton and therefore stimulate synapse formation (Saneyoshi, Wayman et al. 2008). We also reported that CaMKI phosphorylates β-Pix at Ser516 to activate its GEF activity; here we show that phosphorylation at this site is also critical for the leptin-induced current, trafficking of the TrpC channel and filopodia formation. Given that CaMKIγ also colocalizes with TrpC5 channels in axonal growth cones (Davare, Fortin et al. 2009) it will be intriguing to determine whether leptin utilizes the same signaling pathway to activate TrpC4/5 channel-dependent currents in other regions of the brain, such as the hypothalamus.

Finally, we show leptin activates Rac1, a Rho GTPase and important modulator of actin dynamics and synapse formation and maintenance (Nakayama, Harms et al. 2000; Ridley 2001; Penzes, Beeser et al. 2003) and that this activation is required for the leptin-induced current, TrpC1 channel trafficking and
filopodia formation. This is consistent with a known role for Rac1 in vesicular trafficking (Ridley 2001; Symons and Rusk 2003; Chiu, Jensen et al. 2011) as well as neuronal growth factor (NGF)-induced membrane trafficking of TrpC5 in primary hippocampal cultures (Bezzerides, Ramsey et al. 2004).

**Figure 8:** Schematic representation of signaling cascade activated by leptin leading to excitatory synapse formation in hippocampal neurons. LepR, Leptin receptor; Jak2, Janus Kinase2; CaM, Calmodulin; CaMKK, Ca2+/Calmodulin Kinase Kinase; CaMKI, Ca2+/Calmodulin KinaseI; β-Pix, βPak-interacting exchange factor; Rac1, Ras-related C3 botulinum toxin substrate1; Pak1, p21 protein (Cdc42/Rac)-activated kinase 1; TrpC, Transient receptor potential channel C.

In conclusion, our data elucidates a novel molecular pathway by which leptin can activate a TrpC current and induce filopodia formation (summarized in Figure...
8). Since filopodia are thought to be the precursors of mature spines where glutamatergic synapses form, this suggests a mechanism by which leptin can initiate synaptic development and modulate hippocampal function. Leptin treatment increases excitatory synapses on the POMC neurons in the hypothalamus in vivo (Pinto, Roseberry et al. 2004). What remains to be established is whether this pathway is also involved in leptin-induced synapse formation in other brain regions, such as the hypothalamus.

**Methods**

**Drugs & DNA constructs**

Physiologically active synthetic leptin peptide fragment (116-130) was bought from Tocris bioscience and used at 50nM concentration. STO-609 and SKF96365 were purchased from Tocris bioscience and used at 20µM and 3µM concentration respectively. shLepRb, GCTCAGTCTGAGTTCAGTGAC was cloned in the pSUPER vector. ShRNA constructs for CaMKKa, CaMKKβ, CaMKIβ, CaMKIγ, and β-Pix have been previously described in (Wayman, Impey et al. 2006; Saneyoshi, Wayman et al. 2008). Mutants for β-Pix (S516A and DHm) were described in (Saneyoshi, Wayman et al. 2008). Rac1 constructs (shRNA and dominant negative) and dominant negative Pak1 construct was described in (Impey, Davare et al. 2010).

**Hippocampal Cell Culture Preparation**

Hippocampal neurons (2 x 10^5 cells/cm²) were cultured from P1 Sprague-Dawley rats on plates coated with poly-L-lysine from Sigma-Aldrich (molecular weight 300,000). Hippocampal neurons were maintained in Neurobasal A medium from Invitrogen (Carlsbad, CA) supplemented with B27 (Invitrogen) and 0.5mM L-
glutamine and 5mM cytosine-D-arabinofuranoside (Sigma-Aldrich) added at 2 days in vitro. Hippocampal neurons were then cultured a further 3 to 7 days, at which time they were either transfected or treated with various pharmacological reagents as described in Wayman et al. (2008).

**Whole-cell recordings**

Patch-clamp experiments were performed on mRFP-β-actin-transfected cultured DIV8 to DIV9 hippocampal neurons. Transfected cells were visualized with fluorescence (IX-71, Olympus Optical). Recordings were made at room temperature with the membrane potential held at −70 mV in an extracellular solution of 140 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 3 mM CaCl2, 25 mM glucose, and 5 mM HEPES; pH 7.3, 305 to 310 mOsM and with 100 mM Picrotoxin, 1 mM strychnine and 500nM tetrodotoxin included in the external solution. The resistance of patch electrodes ranged from 4.0 to 5.2 MΩ, with an internal solution of: 25 mM CsCl, 100 mM CsCH3O3S, 10 mM phosphocreatine, 0.4 mM EGTA, 10 mM HEPES, 2 mM MgCl2, 0.4 mM Mg-ATP, and 0.04 mM Na-GTP; pH 7.2, 296 to 300 mOsM. Recordings were obtained using a Multiclamp 700B amplifier (Molecular Devices), bessel-filtered at 2 kHz, digitized at 10 kHz through a Digidata 1440A interface (Molecular Devices), and acquired using Clampex 10.2 software (Molecular Devices). Only neurons with a >2 GΩ seal and input resistance >240 MΩ were used and liquid junction potentials were not corrected. Data analysis was performed using Clampfit 10.2 software (Molecular Devices) and Mini-Analysis 6.0 software (Synaptosoft, Decatur, GA).

**Transfection**
For immunoprecipitation, neurons were transfected with the desired constructs such as βPix-myc for βPix phosphorylation experiments and TrpC1-flag for biotinylation experiments along with various shRNAs or dominant negative constructs on DIV5-6 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. This protocol yielded a low 3 to 5% transfection efficiency, but the protein was concentrated using appropriate immunoprecipitation protocols mentioned below.

For spine analysis, neurons were transfected with mRFP-βactin along with various other DNA constructs and, shRNAs on day in vitro (DIV) 6 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. This protocol yielded the desired 3 to 5% transfection efficiency thus enabling the visualization of individual neurons. Expression of fluorescently tagged actin allowed clear visualization transfected neurons and their dendritic spines, because dendritic spines are enriched in actin.

**Western Blot**

Protein samples were collected from hippocampal cultures plated at approximately 4.5 x 10^5 cells/cm². Post-treatment cells were lysed with RIPA buffer (Sigma) augmented with Phosphatase inhibitor cocktail 2 and 3 (Sigma), followed by centrifugation at 14000 rpm for 15min to collect the protein-enriched supernatant. The supernatant collected was mixed with SDS-Loading buffer (Invitrogen) and DTT, boiled for 5min and loaded on a SDS-PAGE (Invitrogen). Western blotting was done using the mentioned antibodies at the following dilution: anti-pCREB (Chemicon, 1:1000), anti-myc (Sigma, 1:5000), anti-Flag (Sigma, 1:1000), anti-phosphoβPix (gift from T. Saneyoshi, 1:200), Avidin-IRDye700 (Rockland,
1:10,000). Blots were imaged using Odyssey Infrared Detection system and analyzed using ImageJ (NIH) Gel Analyzer tool.

**Immunoprecipitation**

For immunoprecipitation, protein samples were collected as described above and proteins of interest immunoprecipitated using the corresponding antibody (anti-myc antibody for βPix IP, anti-flag M2 antibody for TrpC1 IP) for 18 hours at 4°C. The antibody-protein complex was pulled down using Protein-A agarose beads (Upstate) for 4 hours at 4°C. Collected beads were washed with lysis buffer and eluted in 2X SDS>Loading buffer (Invitrogen) at 50°C for 30 minutes. Eluted protein was sonicated and boiled for 5 minutes before loading on a SDS-PAGE.

**Biotinylation**

After leptin stimulation, hippocampal cultures were first washed with ACSF for 20 minutes and then treated with 1mg/ml NHS-LC-Biotin (Pierce) for 30 minutes. Finally, the cells were washed with a Quenching ACSF for 10 minutes. After the biotinylation steps, cells were lysed as described previously and immunoprecipitated for TrpC1-flag using anti-flag M2 antibody (Sigma).

**Statistical Analysis**

Differences in drug effects were tested by repeated measured ANOVA followed by a Turkey’s post-hoc test. One-way ANOVA was used to analyze the protrusion results and significant effects were analyzed by Tukey post hoc test. Numerical data are expressed as means ±S.E.M.

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References


CHAPTER 2

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Abstract

Abnormal hippocampal dendritic development and synaptogenesis are associated with cognitive and emotional disorders. Leptin, a key regulator of energy homeostasis, acts in the hippocampus to enhance cognition and reduce depression and anxiety. Here we show that leptin induces dendritic spines and glutamate synapse formation in hippocampal neurons and that leptin receptors (lepRs) mediate these effects both in vitro and in vivo. Activation of LepR leads to CREB phosphorylation via the Mek/Erk pathway resulting in initiation of CREB-dependent transcription. Expression of microRNA-132, a well-known CREB target and promoter of Rac1 mediated synaptogenesis, increased with leptin and this was required for leptin-induced synaptogenesis. Leptin also suppressed the expression of p250GAP, a miR132 target, and this suppression was also required for leptin effects. In
conclusion, we identify a novel signaling pathway by which leptin increases synaptogenesis, involving CREB-dependent transcription of miR132 and suppression of p250GAP expression.

**Introduction**

Leptin, a 16kD type I cytokine, is a key hormonal regulator of energy homeostasis (Halaas, Gajiwala et al. 1995; Ahima and Flier 2000). In adults, plasma leptin levels are proportionate to white adipose tissue mass (Maffei, Halaas et al. 1995). It decreases food intake in non-obese adults, but has no effect on feeding in neonates (Proulx, Richard et al. 2002). However, leptin levels surge during a crucial developmental period (PND 7-14) that correlates with a period of rapid neuronal growth within the CNS (Ahima, Prabakaran et al. 1998; Ahima, Bjorbaek et al. 1999), suggesting that leptin might function as a neurotrophic signal. Lack of leptin signaling during this key period of neuronal development inhibits the formation of feeding circuits within the hypothalamus (Bouret, Draper et al. 2004) and alters the expression of synaptic proteins in the hippocampus (Ahima, Bjorbaek et al. 1999). Moreover, exogenous administration of leptin to these animals during this critical period partially rescues the development of the feeding circuit and restores expression of some synaptic proteins in the hippocampus.

Leptin receptors (LepR) are expressed in the hypothalamus and the CA1, CA3, dentate gyrus regions of the hippocampus (Mercer, Hoggard et al. 1996; Shanley, O'Malley et al. 2002; Guo, Lu et al. 2012). Leptin regulates hippocampal functions such emotional behaviors, spatial memory formation, long-term potentiation (LTP) and long-term depression (LTD) *in vitro* as well as *in vivo* (Li,

Dendritic spines are actin-rich protrusions on the dendritic shaft that are the primary site of excitatory synaptic inputs on pyramidal neurons (Shepherd 1996). Hippocampal spine number correlates with both cognition and mood, with a low mature spine number associated with depression and cognitive impairment (Irwin, Galvez et al. 2000; Pittenger and Duman 2008; Kasai, Fukuda et al. 2010) suggesting this is a potential mechanism by which leptin could alter these hippocampal functions. Disruption of leptin signaling decreases dendritic spine density within the hippocampus in vivo (Stranahan, Lee et al. 2009) and stimulates the formation of dendritic filopodia in vitro (O'Malley, MacDonald et al. 2007).

Furthermore, leptin has been shown to stimulate formation of excitatory synapses in the arucate nucleus of the hypothalamus (Pinto et al., 2004). LepRs are located in somatodendritic regions, including at synapses (Shanley, O'Malley et al. 2002) and we propose that leptin acts as a neurotrophic factor in the hippocampus to increase synapse formation and stabilization, similar to other neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) (Horch, Kruttgen et al. 1999; Scott and Luo 2001; Chapleau, Larimore et al. 2009). In this current study we determine whether leptin stimulates the formation of stable dendritic spines and functional synapses in the hippocampus and the molecular mechanisms involved.
LepRs are type 1 cytokine receptors and the long isoform (LepRb) can activate multiple signaling cascades, including the PI3K pathway, the Jak/STAT pathway and the Mek/Erk pathway (Fruhbeck 2006). Mek/Erk signaling is required for dendritic development and spine formation (Wu, Deisseroth et al. 2001; Vaillant, Zanassi et al. 2002; Wayman, Impey et al. 2006), making it a likely candidate for mediating leptin’s effects on synaptogenesis. Leptin also activates CREB, an activity dependent transcription factor, in the hippocampus (Zhang and Chen 2008). CREB is an important mediator of dendritic spine formation and hippocampal function (Hansen, Sakamoto et al. 2010; Impey, Davare et al. 2010), in part by regulating the expression of key cytoskeletal proteins and microRNAs (Redmond, Kashani et al. 2002; Wayman, Impey et al. 2006). Here we determine whether leptin induces synaptogenesis and the role of the Mek/Erk pathway, CREB and CREB-regulated genes, such as microRNA-132 (miR132). We identify a critical pathway by which leptin stimulates synaptogenesis which could have implications for lepR signaling in other brain regions.

**Results**

**Leptin receptor signaling is required for normal hippocampal spine formation in vivo**

Using the db/db mouse model that has truncated form of lepRb (Chen, Charlat et al. 1996), we examined whether leptin receptor signaling is required for spine formation in vivo. We used a cell filling approach that allows us to visualize distinct dendritic spines by filling discrete hippocampal pyramidal neurons in coronal brain section with a fluorescent dye (Buhl and Lubke 1989; Karatsoreos, Bhagat et al. 2011). Similar to previous reports (Stranahan, Lee et al. 2009), we observed a
lower spine density in db/db mice compared to their wild-type brethren (Figure 1).
The decrease was observed in both the CA1 pyramidal neurons and the CA3
neurons of the db/db mice when compared to wild type controls.

**Figure 1:** Leptin receptor signaling is required for normal hippocampal spine formation in vivo. (A-B) Hippocampal slices were prepared from P28-32 WT (wild type) or db/db mutant mice. CA1 and CA3 neurons were filled with lucifer yellow to visualize spine density. A, Representative images and B, average spine and filopodia density of WT and db/db mice is shown. Data was analysed using student’s t-test (±SEM, ∗p<0.05, ∗∗p<0.01)

**Leptin increases spine formation via LepRb in organotypic hippocampal slice cultures**

In order to confirm that leptin activation of lepR is important for spine formation, we investigated the effects of leptin on spine formation in the presence and absence of lepR expression and/or signaling. We used hippocampal organotypic slice cultures preparation thus preserving the cellular and morphological organization of the hippocampus (Caeser and Aertsen 1991; Bahr 1995). This allowed us to visualize and manipulate specific populations of hippocampal neurons by biolistically transfecting tomato fluorescent protein expressing construct along with other required constructs (Wayman, Impey et al. 2006; Wayman, Davare et
al. 2008). CA1 and CA3 pyramidal neurons both express LepRb receptors and show significant alterations in their morphological architecture under pathological conditions that affect cognitive abilities and emotional regulation, suggesting either could respond to leptin and potentially mediate its effects (Shepherd 1996; Hajszan, Dow et al. 2009; Marchetti, Tafi et al. 2010).

Figure 2: Leptin increases spine formation in hippocampal organotypic slice cultures via the long form of leptin receptor (LepRb). (A-B) Organotypic slice cultures prepared from P5 WT (wildtype) or db/db mice were transfected with tomato-fluorescent protein on DIV2 and stimulated with leptin on DIV4. Cultures were fixed and imaged on DIV7. A, Representative images and B, average density of total spines, mushroom spines, stubby spines and filopodia per condition is shown. (C-D) Organotypic slice cultures from P5 sprague-dawley rats were treated as described above. C, Representative images and D, average density of total spines, mushroom spines, stubby spines and filopodia per condition
is shown. Data was analyzed using one-way ANOVA followed by Tukey post-hoc analysis (±SEM; ***p<0.001, **p<0.01, *p<0.05).

Leptin increased spine density in both CA1 and CA3 regions of wild-type mice hippocampi but had no effect on cultures prepared from db/db mice hippocampi (Figure 2A). This increase in spine density can be attributed to increase in both stubby and mushroom-type mature spines. Additionally, leptin had no effect on filopodia density in either wild-type or db/db mice. We observed similar results when lepR expression was reduced using a short-hairpin RNA (shRNA) targeted to the long isoform lepRb. Organotypic slice cultures prepared from rat hippocampi were biolistically transfected with tomato, a fluorescent protein, along with shLepRb. Leptin increased mature spine density without affecting filopodia density in both CA1 and CA3 regions and shLepRb transfection blocked this affect (Figure 2B).

**Leptin increases functional synapses in cultured hippocampal neurons**

We next determined whether leptin-induced morphologically-mature spines are functional synapses. Dissociated hippocampal neurons were transfected with mRFP-β-Actin to allow precise visualization and quantitation of dendritic spine size, shape, density and functionality (Figure 3A). Concurrent to the slice cultures, leptin increased the density of mature spines without altering the density of dendritic filopodia in dissociated hippocampal neurons (Figure 3A and C). Here as well, leptin’s effects on spinogenesis requires the long-form of the leptin receptor (LepRb) as multiple shRNAs targeting the long form of the LepRb, but not scrambled
Figure 3: Leptin increases the density of excitatory synapses on dissociated hippocampal neurons. (A-D) Cultured hippocampal neurons were transfected on DIV5 with mRFP-βactin ±shLepR, followed by leptin stimulation on DIV7 until DIV12 when the
cultures were fixed and immunostained with anti-VGLUT antibody. A, representative images displaying mRFP-βactin ±shLepR fluorescence, VGLUT staining and an overlay with mRFP fluorescence in red and VGLUT stain in green. The mRFP fluorescence was used to categorize dendritic protrusions as either filopodia (white arrow) or spines. Spines were further categorized as stubby (red arrow) or mushroom (yellow arrow). B, average percentage juxtaposition (±SEM) between VGLUT puncta and βactin-rich spines, C, average density (±SEM) of total spines, mushroom spines, stubby spines and filopodia and, D, cumulative frequency analyses of spine head-size under control and leptin stimulated conditions ±shLepR . (E-H) Cultured hippocampal neurons were transfected and stimulated as described earlier and mEPSCs were recorded on DIV12. E, representative traces recorded from control and leptin stimulated neurons with and without shLepR transfection. F, frequency, G, amplitude, H, decay time of mEPSCs under control and leptin stimulated conditions ±shLepR. All data was analyzed using one-way ANOVA followed by either Tukey’s (for spine analysis) or Newman-Keuls (for electrophysiology) post-hoc analysis (±SEM, ***p<0.0001, **p<0.001, *p<0.01, #p<0.05 compared to control).

shRNA control constructs, inhibited leptin-induced increases in spine density (Figure3A and C, data not shown). Interestingly, knockdown of lepRb also reduced basal spine formation/stability as neurons transfected with shLepRb have significantly reduced spine density and increased filopodia density compared to the control (Figure3A and C). Leptin caused a shift in spine head size, reflecting the preferential increase in both mushroom and stubby spines (Figure3D), suggesting lepRb signaling drives production and stabilization of more mature spines.

To confirm that the leptin-induced spines formed functional synapses we used two complementary techniques, immunocytochemistry and electrophysiology. Immunostaining for VGLUT1 (Glutamatergic synaptic marker) and synapsin
(general synaptic marker) showed these pre-synaptic markers were found opposite a mRFP-β-Actin labeled spine head approximately 95% of the time (control 92.78±3.183, leptin 90.45±2.537, shLepRb 91.82±4.196, shLepRb+leptin 97.45±4.847 %juxtaposition) (Figure3A and B).

Although leptin stimulation increased the total number of dendritic spines, neither leptin nor shLepRb altered the percent juxtaposition of spines with pre-synaptic terminals. Leptin treatment also increased the frequency of mEPSCs approximately five times above control suggesting that leptin-induced spines form functional synapses (Figure3E and F). In contrast, while the mean amplitude of mEPSCs was significantly increased in some cultures the effect was not seen in the majority of cultures and leptin had no effect on the decay time of the mEPSCs (Figure3G). Targeted knockdown of lepRb completely blocked leptin’s effects to increase mEPSC frequency (Figure3E and F). Together, these data provide strong evidence that leptin stimulates the formation of functional synapses in dissociated hippocampal cultures through activation of the long form of the leptin receptor. Leptin-induced spine formation was blocked by the NMDAR antagonist AP5 (data not shown), suggesting that leptin’s effects on spine formation are also activity dependent.

**Leptin stimulates the phosphorylation and activation of CREB via the Mek/Erk pathway.**

LepRb is a tyrosine-kinase receptor that can initiate different molecular cascades; such as the Jak/STAT pathway, the PI3K pathway and the Mek/Erk/CREB pathway (Tartaglia, Dembski et al. 1995; Hegyi, Fulop et al. 2004; Fruhbeck 2006).
Since we are interested in the chronic effects of leptin we chose to focus on transcription-dependent machinery downstream of the leptin receptor.

**Figure 4:** Leptin increases CREB but not STAT3 phosphorylation and transcription via the Mek/Erk pathway. (A-B) DIV6 hippocampal neurons were stimulated with leptin for various durations from 5 minutes up to 30 minutes. Cells were lysed in RIPA buffer and analysed using SDS-PAGE. A, representative western blot and B, average relative intensity (±SEM) of pCREB and pSTAT3 bands normalized to Erk2 under control and leptin stimulated conditions. (C-D) Cultured hippocampal neurons were transfected on DIV5 with mRFP-βactin ± shLepR, followed by stimulation with or without leptin on DIV7 for 15 minutes until fixed and immunostained with anti-pCREB antibody (1:500, Upstate). Intensity of pCREB staining was measured from 30 neurons per condition in 3 separate experiments. C, representative images showing mRFP-βactin transfection ± shLepR, pCREB staining and an overlay with mRFP fluorescence in red and pCREB stain in green. D, average relative intensity (±SEM) of pCREB staining in transfected neurons under control and leptin
stimulated conditions ±shLepR. (E) DIV 6 hippocampal neurons were treated with or without UO126 (Mek1 inhibitor) for 30 minutes prior to a 15 minutes stimulation with or without Leptin. Cells were lysed in RIPA buffer and analysed using SDS-PAGE. Representative western blot (inset) and average relative intensity (±SEM) of pCREB bands normalized to Erk2 under control and leptin stimulated conditions ±UO126. (F) Cultured hippocampal neurons were transfected on DIV5 with CRE-luciferase and Gal-CREB constructs along with either empty vector or shLepR or MT CREB, followed by stimulation with or without leptin on DIV7 for 4 hours. UO126 was added 30 minutes prior to leptin treatment to indicated samples. Post leptin stimulation cells were lysed in lysis reagent (provided in Promega kit) and total protein concentration was measured using BCA assay (Peirce). Luciferase activity was measures using Promega Luciferase kit. Normalised luciferase activity under various conditions is shown. Data from western blots was analyzed using student’s t-test (**p<0.001). Data for immunostaining and luciferase activity was analyzed using one-way ANOVA followed by Tukey’s post-hoc analysis (**p<0.001, **p<0.01, compared to control).

Similar to certain previous reports (Walker, Long et al. 2007; Garza, Guo et al. 2008; Zhang and Chen 2008), a 15 min leptin treatment preferentially increased Erk2, CREB but not STAT3 phosphorylation in our cultures (Figure4A and B). This increase in CREB phosphorylation was blocked by shLepRb, as detected by immunostaining (Figure4C and D) and pre-treatment with UO126, a Mek1 inhibitor, as detected by western blot (Figure4E). We also confirmed if leptin activated CREB-dependent transcription using a CRE-luciferase assay. Leptin (4 hr treatment) increased CREB-dependent luciferase transcription as is measured by luciferase activity but pre-treatment with UO126 and shLepRb blocked this effect (Figure4F). Most importantly, MT CREB, a mutant CREB form that cannot be phosphorylated at
Ser133 and activated, also blocked leptin’s effects on CREB-dependent transcription suggesting that leptin increases CREB activity and transcription by phosphorylating it at Ser133.

**CREB activity is required for leptin-induced synaptogenesis.**

Activation of CREB and CREB dependent transcription stimulates synaptogenesis and is required for activity-induced spine formation (Impey, Davare et al. 2010). To test whether leptin activation of CREB was required for leptin-induction of spinogenesis we co-transfected either ACREB, a dominant negative form of CREB or shCREB, a short-hairpin RNA that blocks the expression endogenous CREB along with mRFP-β-Actin. Inhibition of CREB by either method completely blocked leptin-induced spine formation (Figure 5A and B) showing that CREB expression and activity is essential. In contrast, ACREB and shCREB transfection alone did not significantly alter spine density compared to control. We next confirmed the requirement of CREB in leptin-induced synapse formation by analyzing the frequency of mEPSCs following inhibition of CREB function. Leptin treated neurons had a significant increase in the frequency but not amplitude or decay time of mEPSCs (Figure 5C, D and E). Expression of ACREB or shCREB completely blocked leptin’s effects on mEPSC frequency (Figure 5C), but had no effect on control levels of spinogenesis.
**Fig5: CREB activity is required for leptin induced synapse formation.** (A-B) Cultured hippocampal neurons were transfected on DIV5 with mRFP-βactin ±ACREB, ±shCREB, followed by stimulation with or without leptin on DIV7 until DIV12 when the cultures were fixed. **A**, representative images and **B**, average density (±SEM) of total spines, mushroom spines, stubby spines and filopodia under control and leptin stimulated conditions ±ACREB, ±shCREB. (C-E) Cultured hippocampal neurons were transfected and stimulated as described earlier and mEPSCs were recorded on DIV12. **C**, frequency, **D**, amplitude, **E**, decay time of mEPSCs.
decay time of mEPSCs under control and leptin stimulated conditions ±ACREB. \( (F-G) \)

Organotypic slice cultures prepared from P5 rats were biolistically transfected on DIV2 with tomato ±ACREB, followed by stimulation with or without leptin on DIV4 until DIV7 when the cultures were fixed. \( F \), representative images and \( G \), average density (±SEM) of total spines, mushroom spines, stubby spines and filopodia under control and leptin stimulated conditions ±ACREB. Data was analyzed using one-way ANOVA followed by either Tukey’s (for spine analysis) or Newman-Keuls (for electrophysiology) post-hoc analysis (***p<0.0001, **p<0.001, *p<0.01, #p<0.05 compared to control).

We found similar results in hippocampal slice cultures as neurons transfected with ACREB did not respond to leptin treatment while control neurons still showed an increase in spine density (Figure5F and G). These results strongly indicate the importance of CREB and CREB-dependent transcription in leptin’s effects on synapse formation in dissociated hippocampal cultures.

**Leptin requires CREB-regulated miR132 transcription and activity for enhancing synapse formation.**

Numerous studies have provided evidence for the central role of CREB in neuronal plasticity but only a few downstream target of this prolific transcription factor have been implicated in this function. Recently, miR132 has emerged as an interesting CREB target that has been shown to be involved in activity-dependent as well as neurotrophic factor-dependent neuronal arborization and synaptogenesis (Vo 2005; Wayman, Davare et al. 2008) Impey et al ). Using real-time qRT-PCR, we determined whether leptin treatment increased the expression of pre- as well as mature-miR132. Leptin increased the transcription of pre-miR132 within 30min and
this increase peaks at 2-4h, with levels returning closer to baseline at 8h in dissociated hippocampal cultures (Figure6A). However, mature miR132 levels increase only modestly after 2-4h and dramatically after 8h of leptin treatment (Figure6A). Additionally, we found that basal levels of mature miR132 are approximately 60% lower in db/db mice compared to wild-type mice (Figure6B). Furthermore, leptin stimulation of pre-miR132 expression was blocked by UO126 pre-treatment, confirming that leptin via the Mek/Erk pathway activates CREB-dependent transcription of miR132 (data not shown). We next determined whether miR132 is required for leptin-induced synapse formation by inhibiting miR132 function by transfecting with anti-sense 2’O-methyl RNA oligonucleotide targeting to miR132 (2’OM-miR132) along with mRFP-β-actin. Transfection of 2’OM-miR132 had no significant effect on the density of dendritic spines under basal conditions but did increase filopodia density (Figure6B). However, leptin failed to increase spine density in 2’OM-miR132 transfected neurons (Figure6B).

We also analyzed mEPSC frequency following miR132 inhibition. As previously demonstrated leptin significantly increased the frequency of mEPSCs. In contrast, neurons transfected with 2’OM-miR132 showed a slight decrease in mEPSC frequency under unstimulated conditions but did not respond to leptin (Figure6C). Inhibition of miR132 activity also blocked leptin’s effects on spine formation in organotypic slice cultures, but again had no effect on basal spine density (Figure6D). These data support the conclusion that leptin induction of miR132 is required for leptin stimulated synaptogenesis.
Figure 6: Leptin induces miR132 transcription to induce spine formation. (A) DIV6 hippocampal neurons were treated with leptin for various durations from 30 minutes to 8 hours. Cells were lysed in triazol and RNA was isolated and converted to cDNA (using Random Hexamers for pre-miR132 or TaqMan RT-primers for mature-mir132). The cDNA samples were then analyzed for changes in pre-miR132 transcript levels (normalized to PPIA) and mature miR132 transcript levels (normalized to U6 snRNA). (B) Hippocampi collected from P42 WT (wildtype) or db/db mice were lysed in triazol and RNA was isolated. cDNA was prepared and analyzed for mature miR132 transcript levels (normalized to U6 snRNA). (C-D) Cultured hippocampal neurons were transfected on DIV5 with mRFP-βactin ±2'OM-miR132, followed by stimulation with or without leptin on DIV7 until DIV12 when the cultures were either fixed and imaged or used for electrophysiological measurements. C, frequency of mEPSC under control and leptin stimulated conditions ±2'OM-miR132. D, average density (±SEM) of total spines, mushroom spines, stubby spines and filopodia
under control and leptin stimulated conditions ±2’OM-miR132. (E) Organotypic slice cultures prepared from P5 rats were biolistically transfected on DIV2 with tomato ±2’OM-miR132, followed by stimulation with or without leptin on DIV4 until DIV7 when the cultures were fixed. Average density (±SEM) of total spines, mushroom spines, stubby spines and filopodia under control and leptin stimulated conditions ±2’OM-miR132 was quantified. RT-PCR data was analyzed using student’s t-test. Spine density and electrophysiological data was analyzed using one-way ANOVA followed by either Tukey’s (for spine analysis) or Newman-Keuls (for electrophysiology) post-hoc analysis (***p<0.0001, **p<0.001, *p<0.05 compared to control).

**p250GAP, a miR132 target mediates leptin-regulated synapse formation.**

MicroRNAs are short RNAs that inhibit the translation of their target mRNA by binding to specific regulatory elements on the mRNA. We recently demonstrated that miR132 regulates activity dependent dendritic growth and synaptogenesis by inhibiting the translation of its target, p250GAP, a Rac GTPase Activating Protein (GAP) (Wayman, Davare et al. 2008). Knockdown of p250GAP by targeted shRNA markedly increased spine density and spine size, the same effect as we report for leptin (Impey, Davare et al. 2010). Therefore, we next tested whether leptin suppressed p250GAP expression and if this was required for leptin-stimulated synaptogenesis. Leptin dramatically reduced p250GAP expression (80% reduction after 24h) via the Mek/Erk pathway (Figure7A and B). Furthermore, expression of mutant p250GAP (MTp250GAP), that is relieved of miR132 down-regulation, inhibited leptin stimulated spinogenesis, supporting the hypothesis that leptin regulates synaptogenesis via miR132-dependent suppression of p250GAP (Figure7C).
**Figure 7: Leptin reduces p250GAP expression to induce spine formation.**

(A-B) DIV6 hippocampal neurons were treated with or without 20µM UO126 (Mek1 inhibitor) for 30 minutes prior to a 24 hour stimulation with or without leptin. Cells were lysed in RIPA buffer and analysed using SDS-PAGE. A, representative western blot and B, average relative intensity (±SEM) of p250GAP bands normalized to Erk2 under control and leptin stimulated conditions ±UO126.

(C) Cultured hippocampal neurons were transfected on DIV5 with mRFP-βactin ±MTp250GAP, ±siRac1, followed by stimulation with or without leptin on DIV7 until DIV12 when the cultures were fixed. Average density (±SEM) of total spines, mushroom spines, stubby spines and filopodia under control and leptin stimulated conditions ±MTp250GAP, ±siRac1 was quantified. (D) Organotypic slice cultures prepared from P5 rats were biolistically transfected on DIV2 with tomato ±MTp250GAP, ±siRac1, followed by stimulation with or without leptin on DIV4 until DIV7 when the cultures were fixed. Average density (±SEM) of total spines, mushroom spines, stubby spines and filopodia under control and leptin stimulated conditions ±MTp250GAP, ±siRac1 was quantified. Data for western blot was analyzed using student’s t-test. Spine data was
P250GAP has been shown to regulate Rac, RhoA and Cdc42 activity by increasing their endogenous GTPase activity (Nakayama, Harms et al. 2000; Nakazawa, Watabe et al. 2003). In DIV7 postnatal hippocampal neurons p250GAP primarily inhibits the activity of Rac1, a central regulator of actin rearrangement important for spinogenesis (Impey, Davare et al. 2010). ShRNA-mediated knockdown of Rac1 inhibited basal and leptin-stimulated mature spine formation and increased filopodia density. This suggests that leptin activation of Rac1 is downstream of the leptin-CREB- miR132 pathway and is required for leptin’s effects on synapse formation. Finally, we also found both MTp250GAP and shRac1 blocked leptin’s effects of spine induction in slice cultures (Figure 7D).

Discussion

Since the discovery of leptin, it has emerged as a prominent indicator of an individual’s metabolic state (Halaas, Gajiwala et al. 1995; Ahima and Flier 2000). However, its ability to modulate diverse systems is evident from widespread expression of leptin and its receptors. In the CNS, lepR mRNA and/or immunoreactivity is found in different brain regions including the CA1/CA3 and dentate gyrus of the hippocampus (Mercer, Hoggard et al. 1996; Elmquist, Bjorbaek et al. 1998). Additionally, leptin mRNA and/or immunoreactivity is also observed in the hippocampus (Ur, Wilkinson et al. 2002). Not only is there a possibility that leptin produced in the hippocampus itself can affect it’s functioning
but leptin present in the plasma has also been shown to enter the hippocampus at saturating concentrations (Banks, Clever et al. 2000). Over the past decade, numerous reports have described leptin’s effects on hippocampal synaptic functioning (LTP, LTD), morphology (actin reorganization, filopodia formation) and related behaviors (cognitive function, depression, anxiety). In this report, we present one mechanism (summarized in model shown in Figure 8) responsible for leptin-induced synaptogenesis that could underlie some of the previously described effects.

**Figure 8: Schematic representation of signaling cascade activated by leptin leading to excitatory synapse formation in hippocampal neurons.**

Dendritic spines, although dynamic in nature, can be stable for at least a year *in vivo* following activity-dependent strengthening (Grutzendler, Kashuri et al. 2002; Trachtenberg, Chen et al. 2002). In fact, changes in spine type, size, density and receptor composition have been associated with cellular correlates of learning and memory i.e. LTP and LTD (Kasai, Fukuda et al. 2010). An increase in
volume/size of the spine head and trafficking of additional AMPA receptors is also observed as spines stabilize following LTP (Kasai, Fukuda et al. 2010). We observed that leptin preferentially increased the density of stubby and mushroom-type spines in both slice and dissociated cultures. It also significantly enlarged the spine head size indicating formation of mature and stable spines similar to spines observed following LTP. Moult et. al. have shown the leptin increases the synaptic trafficking of GluA1 AMPA receptors in hippocampal slices (Moult, Cross et al. 2010). We also observed an increase in frequency of AMPA receptor based mEPSCs indicating that leptin-induced spines are functional synapses with active AMPA receptor population. Together, these data are concurrent with previous reports that demonstrate leptin-induced LTP and requirement of leptin signaling for proper LTP function (Shanley, Irving et al. 2001; Li, Aou et al. 2002; Wayner, Armstrong et al. 2004). Beyond LTP, formation and stabilization of new spines in the cortex have now been directly associated with sensory excitation and motor learning (Hofer and Bonhoeffer 2010). Leptin as well has been shown to have memory enhancing abilities. Bilateral leptin injections into the hippocampus improve retention of hippocampal-dependent learning tasks (Farr, Banks et al. 2006). LepR lacking db/db mice, not only show impaired LTP, but also perform worst at spatial learning tasks (Li, Aou et al. 2002). We predict that lack of lepR signaling in the db/db mice is directly responsible for their learning disabilities, since we observe a lower spine density in db/db animals and an inability of leptin to cause spine formation in slice cultures prepared from db/db hippocampi. Along with this demonstration of leptin’s effects on synapse formation, some reports show it as neuroprotective and important for hippocampal neurogenesis (Shanley, O'Malley et al. 2002; Garza, Guo et al. 2008; Guo, Jiang et
al. 2008; Zhang and Chen 2008), therefore strongly suggesting a new role for leptin as a neurotrophic factor.

The signaling mechanism we lay out in this paper, places CREB-dependent transcription centrally in the leptin signaling cascade leading to synapse formation in the hippocampus. However, the hallmark of leptin signaling has been STAT3 activation, at least in the hypothalamic nuclei (Vaisse, Halaas et al. 1996). Interestingly, leptin lacking ob/ob mice have lower CREB and not STAT3 mRNA levels in the hypothalamus (Duan, Choi et al. 2007). In various studies leptin has been shown to activate both the STAT3 and CREB-dependent transcription (Harris, Aschkenasi et al. 2001; Catalano, Giordano et al. 2009). In the hippocampus, STAT3 is important for cytokine-mediated neuroprotective effects including leptin’s neuroprotective effects against neurotrophic withdrawal and oxidative stress (Guo, Jiang et al. 2008; Oliva, Kang et al. 2012). However, leptin preferentially activates CREB-dependent transcription to accomplish its neuroprotective function in ischemic hippocampus (Zhang and Chen 2008). In neonatal rat hippocampus, intraperitoneal leptin injection preferentially activates CREB but not STAT3 (Walker, Long et al. 2007). We too observed a discriminate activation of CREB over STAT3 in our hippocampal dissociated cultures. Furthermore, this leptin-induced CREB phosphorylation was in fact occurring via lepR activation and this phosphorylation event was important for leptin-induced activation of CREB-dependent transcription. These seemingly disparate results could be explained by the different environmentcontexts the hippocampal neurons were exposed to in these studies. Guo et. al. prepared dissociated cultures from embryonic hippocampi and saw leptin working through STAT3 but not MAPK/CREB.
pathway. From their representative western blot (they presented no quantification of this data) it seems like their cultures had very low phosphorylated/activated STAT3 expression under control conditions. In our cultures, prepared from postnatal hippocampi, basal pSTAT3 expression was relatively high (compare to pCREB baseline expression) probably diminishing the inducibility of STAT3 by leptin. They didn’t explore the role of MAPK/CREB in leptin’s neuroprotective effects \textit{in vivo}. On the other hand, Zhang et. al. explored leptin’s neuroprotective effects in response to global ischemia \textit{in vivo} and found the same signal transduction machinery downstream of leptin as us. They didn’t observe any significant change in STAT3 phosphorylation in their model system.

STAT3 is strongly expressed in the brain but there haven’t been any studies implicating its role in actin reorganization, neuronal morphogenesis and especially spine formation. A recent report shows that STAT3 may be important for NMDA receptor-dependent LTD (Nicolas, Peineau et al. 2012). On the other hand, CREB and its target genes have been widely implicated in dendritic development and synapse formation in response to activity and neurotrophic factor such as BDNF (Redmond, Kashani et al. 2002; Vo 2005; Wayman, Impey et al. 2006; Impey, Davare et al. 2010)(Lesiak et. al. 2012) thus making it a likely target for leptin’s effects of synapse formation. This is exactly what we found. Reducing CREB function with a dominant negative mutant ACREB or inhibiting its expression with a targeted shRNA, blocks leptin from synapse formation in both dissociated as well as slice cultures. Similar to previous reports, ACREB reduced basal spine formation in slice cultures but not in dissociated cultures. It also successfully blocked leptin-induced mEPSC indicating it is important for both leptin-induced changes in synapse
function. CREB phosphorylation and thus activation can be achieved by initiating various second-messenger system such as cAMPK/PKA, Ca\(^{2+}/CaM\), PI3K and MAPK pathway (De Cesare, Fimia et al. 1999). Leptin has been shown to function via the PI3K and MAPK pathways (Fruhbeck 2006). In fact, leptin requires both PI3K and MAPK pathways for its effects of NMDA receptor function in the hippocampus (Shanley, Irving et al. 2001). However, O'Malley et. al. show that leptin acts via the MAPK and not PI3K pathway for actin reorganization (O'Malley, MacDonald et al. 2007). Additionally, the MAPK signaling cascades has itself been implicated in dendritic development and spine formation in many different paradigms; such as activity dependent dendritic development and neurotrophic-factor dependent spine formation (Wu, Deisseroth et al. 2001; Vaillant, Zanassi et al. 2002; Wayman, Impey et al. 2006). Here we show using UO126, a Mek1 inhibitor, that leptin requires the MAPK pathway for CREB phosphorylation and activation of CREB-dependent transcription. We also find that UO126 can block leptin’s effects on spine formation and mEPSC frequency (data not shown).

Downstream of CREB signaling, we report leptin regulation of any microRNA for the first time. The pre-miR132 transcript is an immature form of miR132 that contains two different miRNA transcripts; miR132 and miR212.5p (Vo 2005). We have focused our attentions on miR132 instead of miR212.5p because a recent study reports that miR132 is the predominant miRNA transcribed from this locus (Magill, Cambronne et al. 2010). Interestingly, miR132 knock-out mice show a deficit in novel-object recognition task indicating impaired memory formation (Hansen, Sakamoto et al. 2010). Leptin increased transcription of both pre-miR132 as well as its mature form. Additionally, lepRb lacking db/db had reduced mature
miR132 levels. The mature miR132 can then act on its target mRNAs suppressing their translation. We show that expression of p250GAP, Rac1 inhibitor and miR132 target, reduces 24 hours post-leptin treatment. This miR132 induced suppression of p250GAP is important for leptin-induced synapse formation. The effect of blocking miR132 function using a 2’O-methyl modified miR132 antagonir on basal spine formation in dissociated hippocampal neurons was not as pronounced or significant as previously reported (Impey, Davare et al. 2010). However, 2’OM-miR132 treatment did reduce basal mEPSC frequency in dissociated hippocampal neurons and basal spine formation in slice cultures. Similarly for p250GAP, over-expression of the mutant/miR132 unregulated p250GAP blocked leptin’s effects on synapse formation without affecting basal synapse formation in dissociated cultures but reducing basal spine formation in slice cultures. This variation in the effects of blocking miR132 function or enhancing p250GAP function on basal spine formation between dissociated and slice cultures could be explained by the very distinct environments neurons are exposed to in these preparations. In organotypic slice cultures, neurons maintain their physiological connections with other types of neurons while in dissociated cultures brand new circuits are created on the coverslip.

Finally, with leptin reducing the expression of p250GAP, it was important to look at the role of Rac1. Rac1 is a small GTPase that p250GAP serves to inactivate (Impey, Davare et al. 2010). It is also a central promoter of actin reorganization and spine formation in the hippocampus (Nakayama, Harms et al. 2000). As expected, targeted knock down of Rac1 expression by a shRNA not only eliminated leptin’s effects on spine formation but also reduced basal spine density.
Many reports have described the important role of leptin in learning and memory formation. In this report, we determine a potential mechanism that could be behind those findings. Leptin has been shown to regulate synapse number in the hypothalamus as well to mediate its effects on feeding. We hypothesize an involvement of the mechanism described here in leptin induced synapse formation in the hypothalamus as well. Although, we have focused on leptin, but other hormones have also been implicated in wide array of CNS functions, especially hippocampal synaptic plasticity (Moult and Harvey 2008). This work, along with many others, suggests a vital role for peripherally produced signals in regulating more than endocrine functions. Hormones are messengers carrying information about changes in bodily functions in response to external or internal agents. Therefore, it is plausible to expect the brain to consider this information in its cognitive machinery and create adaptive behaviors.

**Methods**

**Animals**

B6.BKS(D)-Leprdb/J and BKS.Cg-Dock7m +/- Leprdb/J, heterozygous db/db mice breeding pairs were ordered from Jackson Laboratories. They were housed on a 12 h light/dark cycle at ambient temperature. Chow and water were provided ad libitum. All animal procedures were conducted with the approval of the Institutional Animal Care and Use Committee. Animals were genotyped using an Endpoint Allelic discrimination protocol available on Jackson Laboratories website. Wildtype pups obtained from the heterozygous db/db mice pairs or from C57/Bl breeding pairs were used as wildtype controls in all designated experiments. Leptin receptor
mutant pups obtained from the heterozygous db/db mice pairs were used as db/db in all designated experiments.

**Cell Filling**

P28-30 mice were used for the cell filling experiments. The protocol followed was as described in Karatsoreos et al. (Karatsoreos, Bhagat et al. 2011).

**Drugs & DNA constructs**

Physiologically active synthetic leptin peptide fragment (116-130) was bought from Tocris bioscience and used at 50nM concentration. UO126 was bought from Calbiochem and used at 20µM concentration. shLepRb, GCTCACTGTCTGTTCAGTGAC was cloned in the pSUPER vector. ACREB, shCREB, 2’OM-miR132, MTp250GAP, shRac1 are described previously (Impey, Davare et al. 2010).

**Hippocampal Slice Culture Preparation & Transfection**

Organotypic hippocampal slices from P5 Sprague-Dawley rats, C57/Bl mice or C57/Bl db/db mice were cultured for 3 days as described (Barria and Malinow, 2002). To visualize dendritic arbors, slices were transfected with pCAGGS-Tomato using a Helios Gene Gun (BioRad), according to the manufacturer’s protocol. Following transfection, slices were allowed to recover for 24h before stimulation with 50nM leptin for 2 days. Slices were fixed, mounted, and imaged using a confocal microscope. Dendritic spine and filopodia density was measured as described below.

**Hippocampal Cell Culture Preparation & Transfection**

Hippocampal neurons (2 x 10^5 cells/cm^2) were cultured from P1 Sprague-Dawley rats on plates coated with poly-L-lysine from Sigma-Aldrich (molecular weight 300,000). Hippocampal neurons were maintained in Neurobasal A medium from
Invitrogen (Carlsbad, CA) supplemented with B27 (Invitrogen) and 0.5mM L-glutamine and 5mM cytosine-D-arabinofuranoside (Sigma-Aldrich) added at 2 days in vitro. Neurons were transfected with mRFP-βactin along with various other DNA constructs, shRNAs and 2’OM-antagomers on day in vitro (DIV) 6 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. This protocol yielded the desired 3 to 5% transfection efficiency, thus enabling the visualization of individual neurons. Higher efficiencies obscured the dendritic arbor of individual neurons. Expression of fluorescently tagged actin allowed clear visualization transfected neurons and their dendritic spines, because dendritic spines are enriched in actin.

**Spine Quantification**

On DIV7, the cells were treated with 50nM leptin (as described in the text) added to media. In case of UO126 pre-treatment, cultures were incubated with 20µM UO126 (Calbiochem) added to the media for 30min before leptin stimulation. On DIV12, the neurons were fixed (4% paraformaldehyde in PHEMS buffer, pH 7.4) for 20min at room temperature and mounted on glass slides using elvanol. Slides were dried for at least 20h at 4°C, and fluorescent images were obtained with Slidebook 5.0 Digital Microscopy Software driving an Olympus IX81 inverted confocal microscope (Olympus Optical, Tokyo, Japan) with a 60X oil immersion lens, numerical aperture 1.4, and resolution 0.280µm. Dendritic spine and filopodia density was measured on primary and secondary dendrites at a distance of at least 100 µm from the soma. Two to five dendrites, each at least 50µm in length, from at least 25 neurons were analyzed for each data point reported. Each experiment was repeated at least three times using independent culture preparations. Dendrite length was
determined using ImageJ 1.41o (National Institutes of Health, Bethesda, MD) and the neurite tracing program Neuron J (Meijering et al., 2004). Spines and filopodia were manually counted. Filopodia were identified as short dendritic protrusions with a long thin neck with or without a small head of diameter less than twice the diameter of the neck. Spines were identified as small protrusions with actin-enriched spherical head connected to a dendrite via a thin neck. The head diameter is at least thrice the diameter of the neck. Spines were further characterized as either stubby or mushroom-type depending on the neck length. Stubby spines have a short or non-existent neck, while mushroom spines have a more discrete neck.

**Immunocytochemistry**

Transfected neurons were treated and fixed as described above. After fixation, cells were rinsed in PBS and permeabilized with 0.1% Triton X-100 detergent (Bio-Rad Laboratories, Hercules, CA), followed by two rinses in PBS, and blocked with 0.5% fish gelatin in PBS for 2h. Cells were rinsed with PBS again, followed by a 24h incubation period with anti-VGLUT1 (Synaptic Systems, Goettingen, Germany) or anti-synapsin (Synaptic Systems) or anti-pCREB (Chemicon), following the manufacturer’s protocol, at 4°C. Then, cells were rinsed twice with PBS, incubated in Alexa Fluor 488 goat-anti-mouse IgG following the manufacturer’s protocol (Invitrogen) for 2 h at room temperature, rinsed again with PBS, and mounted with elvanol. Imaging for pre-synaptic markers was performed as described in the spine quantification section since spine measurements and pre-synaptic puncta juxtaposition were performed on the same cultures. Each spine on a dendritic length being analyzed was also analyzed for pre-synaptic puncta juxtaposition.
Imaging for pCREB staining was performed using Slidebook 5.0 Digital Microscopy Software driving an Olympus IX81 inverted confocal microscope (Olympus Optical, Tokyo, Japan) with a 20X lens, numerical aperture 0.5, and resolution 0.33µm. Using the ImageJ software, pCREB staining intensity was measured in the soma of transfected neurons.

**Western Blot**

Protein samples were collected post-treatment by lysing the cells with RIPA buffer (Sigma) augmented with Phosphatase inhibitor cocktail 2 and 3 (Sigma), followed by centrifugation at 14000 rpm for 15min to collect the protein-enriched supernatant. The supernatant collected was mixed with SDS-Loading buffer (Invitrogen) and DTT, boiled for 5min and loaded on a SDS-PAGE (Invitrogen). Western blotting was done using the mentioned antibodies at the following dilution: anti-pCREB (Chemicon, 1:1000). Blots were imaged using Odyssey Infrared Detection system and analyzed using ImageJ (NIH) Gel Analyzer tool.

**Real-time qRT-PCR**

RNA samples were collected post-treatment by lysing the cells with triazol (Invitrogen) using manufacturer’s protocol. For pre-miR132 detection, isolated RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) using random primers and used for qRTPCR. RT-PCRs (25µl) contained 12.5µL of Platinum qPCR Supermix (Invitrogen), 0.125mM primer (IDT), 50 X SYBR Green (Invitrogen), and 1000 X Flourescein. All qRT-PCR was run on BioRad icycler for one cycle at 50°C for 2min and 95°C for 2min, and 30–50 cycles at 95°C for 10 s, and 68°C for 45 s. All standard curves had an R² of at least 0.995, were composed of a minimum of 4 points, and were linear for at least 3 orders of magnitude. To
avoid plateau effects, the C<sub>t</sub> was always positioned in the logarithmic component of the sigmoid fluorescence curve. The C<sub>t</sub> was selected based solely on the maximal linearity of standard curve. RT-PCR data were normalized to PPIA cDNA levels also detected by real-time PCR (other house-keeping genes showed similar results). The following primers were used: RT miR132-precursor-1 CTCCGGTTCCCACAGTAACAA, RT miR132-precursor-2 CCGCGTCTCCAGGGCAAC, RT PPIA-1 TTTGGGAAGGTGAAA GAAGG, PPIA-2 ACAGAAGGAATGGTTTGATGG.

For mature miR132 detection, isolated RNA was reverse transcribed for miR132 and U6 snRNA using Applied Biosystems TaqMan RT MicroRNA kit. TaqMan assay was also used to detect mature miR132 and U6 snRNA levels in the samples. P42 mice were used for mature miR132 detection.

**Whole-Cell Recordings**

Patch-clamp experiments were performed on mRFP-βactin-transfected cultured hippocampal neurons with PBS (vehicle control) or 1 pM Nle 1 -AngIV pretreatment. Recordings were made on DIV12 to DIV14. The culture medium was exchanged by an extracellular solution containing 140 mM NaCl, 2.5mM KCl, 1 mM MgCl<sub>2</sub>, 3 mM CaCl<sub>2</sub>, 25 mM glucose, and 5 mM HEPES; pH was adjusted to 7.3 with KOH, and osmolality was adjusted to 310 mOsM. Cultures were allowed to equilibrate in a recording chamber mounted on an inverted microscope (IX-71; Olympus Optical) for 30 min before recording. Transfected cells were visualized with fluorescence (Olympus Optical). Recording pipettes were pulled (P-97 Flaming/Brown micropipette puller; Sutter Instrument Company, Novato, CA) from standard-wall borosilicate glass without filament (o.d. = 1.5 mm; Sutter Instrument Company). The pipette-to-bath d.c. resistance of patch electrodes ranged from 4.0 to 5.2 MΩ,
and they were filled with an internal solution of the following composition: 25 mM CsCl, 100 mM CsCH$_3$O$_3$S, 10 mM phospho-creatine, 0.4 mM EGTA, 10 mM HEPES, 2 mM MgCl$_2$, 0.4 mM Mg-ATP, and 0.04 mM Na-GTP; pH was adjusted to 7.2 with CsOH, and osmolality was adjusted to 296 to 300 mOsM. Miniature EPSCs (mEPSCs) were isolated pharmacologically by blocking GABA receptor chloride channels with picrotoxin (100 µM; Sigma-Aldrich), blocking glycine receptors with strychnine (1 µM; Sigma-Aldrich), and blocking action potential generation with tetrodotoxin (500 nM; Tocris Bioscience, Ellisville, MO). Recordings were obtained using a Multiclamps 700B amplifier (Molecular Devices, Sunnyvale, CA). Analog signals were low-pass Bessel-filtered at 2 kHz, digitized at 10kHz through a Digidata 1440A interface (Molecular Devices), and stored in a computer using Clampex 10.2 software (Molecular Devices). The membrane potential was held at -70 mV at room temperature (25°C) during a period of 0.5 to 2 h after removal of the culture from the incubator. Liquid junction potentials were not corrected. Data analysis was performed using Clampfit 10.2 software (Molecular Devices) and Mini-Analysis 6.0 software (Synaptosoft, Decatur, GA). The criteria for a successful recording included an electrical resistance of the seal between the outside surface of the recording pipette and the attached cell >2 GΩ and neuron input resistance >240 MΩ. The mEPSCs had a 5-min recording time.

**CRE-luciferase Assay**

Luciferase activity was measured using a Promega Luciferase assay kit (E1500) and Perkin Elmer VictorX Luminometer.

**Statistical Analyses**
One-way ANOVA was used to analyze the dendritic spine results and significant
effects were analyzed by Tukey post hoc test. Multiple comparisons of
electrophysiological results were made using a one-way ANOVA followed by a
Newman-Keuls post hoc test with a level of significance set at $p < 0.05$. Numerical
data are expressed as means ±S.E.M.

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References


CHAPTER 3
Kruppel-like factor 4 regulates leptin-induced excitatory synaptogenesis in hippocampal neurons.


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Abstract

Kruppel-like factor4 (KLF4) is a transcription factor first identified in the gut and skin epithelium. We found its expression to be highly up-regulated in various high-throughput genome-wide screens conducted on bicuculine-treated neurons, brain derived neurotrophic factor (BDNF)-treated neurons and leptin-treated neurons. In this paper we test the hypothesis that KLF4 increases glutamatergic synapse formation in response to neurotrophic factor stimulation in hippocampal neurons. We found that over-expressing KLF4 in hippocampal cultures increases spine formation, especially mature stubby and mushroom-type spines and increases the frequency of miniature excitatory post-synaptic currents (mEPSCs), consistent with the formation of functional excitatory synapses. The hormone leptin, which we recently showed increases synaptogenesis in hippocampal neurons, transiently increases KLF4 mRNA expression 1 hour post-treatment and KLF4 protein levels 2
hours post-treatment. This increase in KLF4 transcription is required for leptin-induced synapse formation since reducing KLF4 expression using a targeted shRNA inhibits leptin-stimulated spine formation.

**Introduction**

Kruppel-like factor4 (KLF4, aliases GKLF, EZF) is an important transcription factor in the field of stem cell research. In fact it is one of the 4 transcription factors that are sufficient for inducing pluripotency in fibroblast cells (Takahashi and Yamanaka 2006). It was first identified by two separate groups in the gut and skin epithelium (Garrett-Sinha, Eberspaecher et al. 1996; Shields, Christy et al. 1996). It is a zinc-finger transcription factor that was initially thought to be a negative regulator of cellular proliferation (Shields, Christy et al. 1996). However, numerous studies have shown that the effects of KLF4 on different cellular processes are context dependent. KLF4 acts as a tumor suppressor in case of colorectal cancer (Zhao, Hisamuddin et al. 2004; Xu, Lu et al. 2008), esophageal cancer (Wang N 2002) and leukemia (Yasunaga, Taniguchi et al. 2004). But it can also be an oncogene as in case of breast (Foster, Frost et al. 2000) and squamous cell cancer (Huang, Liu et al. 2005). Furthermore, it is both a transcriptional activator as well as a transcriptional repressor (Evans and Liu 2008). It can directly bind to the CACCC motif and activate transcription or interact with HDAC to repress transcription. The complexity of transcriptional regulation by KLF4 has made it an intriguing factor in stem cell research. In this paper, we focus KLF4 effects on post-mitotic, differentiated cells specifically hippocampal neurons.
In the CNS, KLF4 expression rises significantly in retinal ganglion cells of post-natal animal and in cortical cells in response to neuronal activity (Moore, Blackmore et al. 2009; Zhu, Tai et al. 2009). Conversely, it tends to decrease in neural stem cells of a post-natal animal (Qin, Liu et al. 2011). According to some studies, in early developmental stages, KLF4 acts a negative regulator of CNS development. Transgenic mice overexpressing KLF4 in the neural stem cells and astrocytes develop hydrocephalus and have decreased neurogenic activity (Qin, Liu et al. 2011). KLF4 overexpression also decreases radial migration and layering of cortical neurons (Qin and Zhang 2012). However, we have identified KLF4, through various high-throughput genome-wide screens, as an important target for neurotrophic factors such as leptin and brain derived neurotrophic factor (BDNF) that are important for neuronal development and excitatory synapse formation (see chapter 1&2 for leptin) (Chapleau, Larimore et al. 2009; Svitkina, Lin et al. 2010). As is clear from the research done in stem cells, KLF4 effects are highly context dependent. Since KLF4 expression increases with neurotrophic factor treatment, it is plausible that KLF4 mediates some effects of these factors such as increasing excitatory synapse formation. In retinal ganglion cells, KLF4 inhibits axonal regeneration post injury (Moore, Blackmore et al. 2009). This suggests a possible role for KLF4 as a developmental switch in neuronal development. Early in development neurons select an axonal projection, then they develop a dendritic arbor, and synapse formation follows dendritic arborization and increasing spontaneous neuronal and neurotrophic activity. This leads to our hypothesis, that unlike axonal growth, KLF4 increases excitatory synapse formation in response to stimulation by leptin, a neurotrophic factor, in neurons. We use primary
hippocampal cultures as our model system to study synapse formation. In case of hippocampal pyramidal neurons excitatory synapses occur on morphologically distinct structures called the dendritic spine (Harris and Kater 1994). Dendritic spines are classified into three categories based on their shape; namely stubby, mushroom and filopodia (Nimchinsky, Sabatini et al. 2002). Stubby and mushroom-types spines are considered developmentally more mature than filopodia.

Results

Leptin increases KLF4 expression in hippocampal neurons

KLF4 is an activity-regulated transcription factor in the CNS. In this paper, we report the first instance of it being regulated by a neurotrophic factor leptin. We identified KLF4 as a highly up-regulated gene through various high-throughput screens including a leptin RNAseq. We confirmed that leptin does increase KLF4 mRNA transcription in cultured hippocampal neurons using qRT-PCR. Leptin increases KLF4 transcription robustly but only transiently. A significant 12.72±1.81 fold change in KLF4 mRNA levels (normalized to housekeeping gene PPIA) was seen 1hour post leptin treatment, which sustained till 2hours post treatment but then dropped to control levels at 4hours and beyond (Figure1A). This indicates that KLF4 mRNA transcription is tightly regulated. In fact, KLF4 protein expression is also tightly controlled. Although we saw a greater than 12 folds change in KLF4 mRNA transcript levels with leptin treatment, we only observed a 10% change in KLF4 protein levels. KLF4 protein expression was very strong even under control conditions in hippocampal neurons (Figure1B). 2hour post leptin treatment we saw the most significant 10% increase in KLF4 expression (Figure1B and C). This
increase sustained till 8 hours post-treatment, however it became more variable (Figure 3C).

**Figure 1:** Leptin increases KLF4 expression in hippocampal neurons. **(A)** DIV6 hippocampal neurons were treated with leptin for specified duration and processed for real time-qRT-PCR detection of KLF4. Average relative fold change in KLF4 mRNA (normalized to PPIA) is shown. **(B–C)** Cultures were stimulated by leptin on DIV6 for specified duration until fixed and immunostained for KLF4 and MAP2B. **B,** representative images showing KLF4 (red) and MAP2B (green) staining. **C,** relative intensity of KLF4 staining in MAP2B stained neurons. RTPCR data was analyzed using student’s t-test and immunostaining data was analyzed using ANOVA followed by Tukey post-hoc analysis (±SEM, ***p<0.001, **p<0.01, *p<0.05).

**KLF4 increases synaptogenesis in hippocampal neurons**

Since we did observe a significant increase in KLF4 expression with leptin stimulation, we wanted to confirm if KLF4 overexpression in itself has an affect similar to leptin on synapse formation. Dissociated cultured primary hippocampal neurons were transfected with mRFP-β-actin to visualize spine density along with KLF4 expressing construct on DIV6, since spines are enriched with actin. Some control neurons were stimulated with leptin on DIV7 to represent a positive control.
Spines were classified into stubby and mushroom. Filopodia density was also measured. Filopodia were identified as short dendritic protrusions with a long thin neck with or without a small head of diameter less than twice the diameter of the neck. Spines were identified as small protrusions with actin-enriched spherical head connected to a dendrite via a thin neck. The head diameter is at least thrice the diameter of the neck. Spines were further characterized as either stubby or mushroom-type depending on the neck length. Stubby spines have a short or non-existent neck, while mushroom spines have a more discrete neck.

KLF4 over expression increased spine density similar to leptin stimulation (Figure 2A and B). An increase in both stubby and mushroom type mature spines but no change in filopodia density was observed.

**Figure 2: KLF4 increases synaptogenesis in hippocampal neurons.** (A-C) Cultures were transfected with mRFP-βactin ±KLF4, followed by leptin stimulation on DIV7. Treated
cultures were fixed on DIV12 and immunostained with anti-VGLUT. \textbf{A}, Average spine and filopodia density, \textbf{B}, representative images, \textbf{C}, percentage correlation between the pre- and post-synaptic puncta under control, leptin stimulated and KLF4 transfected conditions is shown. \textbf{(D)} Cultures were transfected and stimulated as described earlier and mEPSCs were recorded. Average frequency of mEPSCs (Hz) under control, leptin stimulated and KLF4 transfected conditions is shown. \textbf{(E,F)} Hippocampal organotypic slice cultures prepared from P5 rats were biolistically transfected on DIV2 with tomato ±KLF4, followed by stimulation with Leptin on DIV4 until DIV7 when the cultures were fixed. \textbf{E}, Average spines and filopodia density and \textbf{F}, representative images under control, leptin stimulated and KLF4 transfected conditions is shown. Spine density data was analyzed using Kruskal-Wallis non-parametric ANOVA followed by either Dunn’s post-hoc analysis. Electrophysiology data was analyzed using ANOVA followed by Newman-Keuls post-hoc analysis (±SEM, ***p<0.001, **p<0.01, *p<0.05 compared to control).

In order to confirm that KLF4-induced spines were functional synapses we used two different confirmatory techniques. Neurons transfected with mRFP-βactin were also stained with VGLUT, a pre-synaptic marker. Juxtaposition of mRFP puncta with VGLUT-stained puncta was considered a site for functional synapse. KLF4 overexpressing neurons tended to have lower juxtaposed puncta however this was not significant (93.88±1.54%, control; 86.3±2.92%, KLF4) (Figure2B and C). Next, we used electrophysiology to measure mEPSC from KLF4 overexpressing neurons. Increase in frequency of mEPSC is correlated with increased synapse number. We found that KLF4 overexpression increased the frequency of mEPSC without affecting the amplitude and decay time when compared to control transfected neurons (Figure 2D). However, the increase in mEPSC frequency in KLF4 over-expressing neurons was significantly lower than leptin-treated neurons (p<0.01) (Figure 2D).
We observed a high culture to culture variation in the effects of KLF4. In one culture, KLF4 failed to increase mEPSC frequency while leptin treated neurons had a significant increase. In 4 cultures, KLF4 increased mEPSC frequency 200% above control levels but 32% below leptin levels. Finally, in 2 cultures, KLF4 increased mEPSC frequency 500% above control levels but 22% below leptin levels. While KLF4 increased mEPSC frequency in most cultures except one, they tended to below leptin levels. This data does not completely agree with the spine density data from dissociated cultures since we saw KLF4 phenocopy leptin’s effects on spine formation but not on mEPSC frequency. In order to clarify this discrepancy, we decided to use a more intact culture preparation that is considered to imitate physiological environment. In organotypic slice cultures, KLF4 overexpression increased density of mature spines, stubby and mushroom, but filopodia density compared to controls (Figure2E and F). However this increase was still significantly lower than leptin-induced increase in spine density. Therefore, in a more intact preparation, we find that KLF4 doesn’t increase spine density as strongly as leptin and this data complies better with the electrophysiology data.

**KLF4 is required for leptin-induced synaptogenesis**

Although, KLF4 may not be as strong as a driver of synapse formation as leptin, we still wanted to test if it was required for leptin-induced synapse formation. We used a shRNA targeted to KLF4 that reduces KLF4 expression by 80% (data not shown). Dissociated cultured primary hippocampal neurons were transfected with mRFP-βactin to visualize spine density along with shKLF4 expressing construct on DIV6, followed by leptin stimulation on DIV7.
Figure 3: KLF4 is required for leptin-induced synaptogenesis. (A-B) Cultures were transfected with mRFP-βactin ±shKLF4 on DIV5, followed by leptin stimulation on DIV7 and fixed on DIV12. A, Average spine and filopodia density, B, representative images under control and leptin stimulated conditions ±shKLF4 are shown. (C) Cultures were transfected and stimulated as described earlier and mEPSCs were recorded. Average frequency of mEPSCs (Hz) control and leptin stimulated conditions ±shKLF4 is shown. (D) Hippocampal organotypic slice cultures prepared from P5 rats were biolistically transfected on DIV2 with tomato ±shKLF4, followed by stimulation with leptin on DIV4 until DIV7 when the cultures were fixed. Average spines and filopodia density under control and leptin stimulated conditions ±shKLF4 are shown. Spine density data was analyzed using Kruskal-Wallis non-parametric ANOVA followed by either Dunn’s post-hoc analysis. Electrophysiology data was
analyzed using ANOVA followed by Newman-Keuls post-hoc analysis (±SEM, **p<0.01, *p<0.05 compared to control).

Leptin treatment significantly increased spine density of both stubby and mushroom type mature spines above controls (Figure 3A and B). However, neurons transfected with shKLF4 were unable to respond to leptin and therefore has no increase in spine density. Instead, shKLF4 transfected neurons had a significant decrease in spine density when compared to controls (Figure 3A and B). This decrease was specifically observed in mushroom type spine density. Reducing KLF4 expression also inhibited leptin’s effect on frequency of mEPSC (Figure 3C). While leptin treatment increased mEPSC frequency, shKLF4 transfection blocked this effect. We further confirmed this result in organotypic slice cultures and found that shKLF4 transfection tended to decrease spine density below control levels however not significantly. Leptin increased spine density in these cultures but this effect was blocked by shKLF4 transfection.

**Discussion**

Dendritic spines are the site of excitatory synapses on hippocampal pyramidal neurons. Transcriptional factors such as CREB, MEF2 and NPAS4 are widely studied for their regulation of synapse formation (Barbosa, Kim et al. 2008; Lin, Bloodgood et al. 2008; Impey, Davare et al. 2010). In this paper, we describe KLF4 as another important regulator of neurotrophic-factor dependent excitatory synapse formation.
Hippocampal neurons in culture follow a defined developmental pattern (Dotti, Sullivan et al. 1988). An axon is selected within hours of culturing and by DIV6 neurons have a dendritic arbor and start to develop spontaneous activity. This seems to be appropriate time and context for a neurotrophic factor to successfully enhance KLF4 expression. Leptin increases both transcription and translation of KLF4 gene in DIV6 hippocampal neurons. In addition to leptin, other cytokines such as leukemia inhibitory factor (LIF) (Qin and Zhang 2012) and growth factors such as hepatocyte growth factor (HGF) (Lai, Wu et al. 2012) also induce KLF4 expression. The MAPK (Mek/Erk1/2) pathway has been implicated in mediating the effects of HGF on KLF4 transcription (Lai, Wu et al. 2012). Leptin is a strong activator of the MAPK pathway and could increase KLF4 transcription via this cascade (see chapter2). Additionally, leptin could activate the Mek/Erk5 pathway to increase KLF4 expression as well. Overexpression of Erk5 increases KLF4 expression in endothelial cells (Ohnesorge, Viemann et al. 2010; Clark, Jensen et al. 2011).

Since leptin increases KLF4 expression, we determined the role of KLF4 in synapse formation. KLF4 overexpression phenocopied leptin’s affects on spine formation in dissociated hippocampal cultures. However, it didn’t increase spine density to the same level as leptin in organotypic slice cultures. KLF4 is a tightly regulated transcription factor as is evident from the fact although leptin increases KLF4 transcription by 1200%, the change in protein level is only 10%. In a more intact preparation increasing KLF4 protein levels using DNA constructs might suffer from endogenous regulatory obstacle. Leptin acts via the leptin receptor which belongs to the family of cytokine receptors (Fruhbeck 2006). The leptin receptor
can activate many different signaling cascades and therefore inspite of poor KLF4 activation, it can still strongly drive spine formation. It is important to note here that although KLF4 didn’t increase spine density to the same level as leptin in slice cultures, it still significantly increase spine density by 50% above control. Additionally, the requirement of KLF4 in leptin-induced as well as basal synapse formation is clear from the effects of reducing KLF4 expression by shRNA. Using this approach, we found shKLF4 transfection reduced spine density significantly below control levels in dissociated cultures. It also successfully blocked leptin’s effects on synapse formation. Together these data present KLF4 as an important transcription factor is regulation of synapse formation.

**Methods**

**Drugs & DNA constructs**

Physiologically active synthetic leptin peptide fragment (116-130) was bought from Tocris bioscience and used at 50nM concentration. shKLF4, GCCAGAGGAGCCCAAGCCAAAG was cloned in the pSUPER vector.

**Hippocampal Slice Culture Preparation & Transfection**

Organotypic hippocampal slices from P5 Sprague-Dawley rats were cultured for 3 days as described (Barria and Malinow, 2002). To visualize dendritic arbors, slices were transfected with pCAGGS-Tomato using a Helios Gene Gun (BioRad), according to the manufacturer’s protocol. Following transfection, slices were allowed to recover for 24h before stimulation with 50nM leptin for 2 days. Slices were fixed, mounted, and imaged using a confocal microscope. Dendritic spine and filopodia density was measured as described below.
**Hippocampal Cell Culture Preparation & Transfection**

Hippocampal neurons (2 x 105 cells/cm²) were cultured from P1 Sprague-Dawley rats on plates coated with poly-L-lysine from Sigma-Aldrich (molecular weight 300,000). Hippocampal neurons were maintained in Neurobasal A medium from Invitrogen (Carlsbad, CA) supplemented with B27 (Invitrogen) and 0.5mM L-glutamine and 5mM cytosine-D-arabinofuranoside (Sigma-Aldrich) added at 2 days in vitro. Neurons were transfected with mRFP-βactin along with various other DNA constructs, shRNAs and 2’OM-antagomers on day in vitro (DIV) 6 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. This protocol yielded the desired 3 to 5% transfection efficiency, thus enabling the visualization of individual neurons. Higher efficiencies obscured the dendritic arbor of individual neurons. Expression of fluorescently tagged actin allowed clear visualization transfected neurons and their dendritic spines, because dendritic spines are enriched in actin.

**Spine Quantification**

On DIV7, the cells were treated with 50nM leptin (as described in the text) added to media. On DIV12, the neurons were fixed (4% paraformaldehyde in PHEMS buffer, pH 7.4) for 20min at room temperature and mounted on glass slides using elvanol. Slides were dried for at least 20h at 4°C, and fluorescent images were obtained with Slidebook 5.0 Digital Microscopy Software driving an Olympus IX81 inverted confocal microscope (Olympus Optical, Tokyo, Japan) with a 60X oil immersion lens, numerical aperture 1.4, and resolution 0.280µm. Dendritic spine and filopodia density was measured on primary and secondary dendrites at a distance of at least 100 µm from the soma. Two to five dendrites, each at least 50µm in length, from at
least 25 neurons were analyzed for each data point reported. Each experiment was repeated at least three times using independent culture preparations. Dendrite length was determined using ImageJ 1.41o (National Institutes of Health, Bethesda, MD) and the neurite tracing program Neuron J (Meijering et al., 2004). Spines and filopodia were manually counted.

Immunocytochemistry

Transfected neurons were treated and fixed as described above. After fixation, cells were rinsed in PBS and permeabilized with 0.1% Triton X-100 detergent (Bio-Rad Laboratories, Hercules, CA), followed by two rinses in PBS, and blocked with 0.5% fish gelatin in PBS for 2 hours. Cells were rinsed with PBS again, followed by a 24 hours incubation period with anti-VGLUT1 (Synaptic Systems, Goettingen, Germany) or anti-KLF4 (SCBT) and anti-MAP2 (Sigma), following the manufacturer’s protocol, at 4°C. Then, cells were rinsed twice with PBS, incubated in Alexa Fluor IgG following the manufacturer’s protocol (Invitrogen) for 2 hours at room temperature, rinsed again with PBS, and mounted with elvanol. Imaging for pre-synaptic markers was performed as described in the spine quantification section since spine measurements and pre-synaptic puncta juxtaposition were performed on the same cultures. Each spine on a dendritic length being analyzed was also analyzed for pre-synaptic puncta juxtaposition. Imaging for KLF4 staining was performed using Slidebook 5.0 Digital Microscopy Software driving an Olympus IX81 inverted confocal microscope (Olympus Optical, Tokyo, Japan) with a 20X lens, numerical aperture 0.5, and resolution 0.33 µm. Using the ImageJ software, KLF4 staining intensity was measured in the soma of transfected neurons.

Real-time qRT-PCR
RNA samples were collected post-treatment by lysing the cells with triazol (Invitrogen) using manufacturer’s protocol. Isolated RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) using random primers and used for qRTPCR. RT-PCRs (25µl) contained 12.5µL of Platinum qPCR Supermix (Invitrogen), 0.125mM primer (IDT), 50 X SYBR Green (Invitrogen), and 1000 X Flourescein. All qRT-PCR was run on BioRad icycler for one cycle at 50°C for 2min and 95°C for 2min, and 30–50 cycles at 95°C for 10 s, and 68°C for 45 s. All standard curves had an R2 of at least 0.995, were composed of a minimum of 4 points, and were linear for at least 3 orders of magnitude. To avoid plateau effects, the Ct was always positioned in the logarithmic component of the sigmoid fluorescence curve. The Ct was selected based solely on the maximal linearity of standard curve. RT-PCR data were normalized to PPIA cDNA levels also detected by real-time PCR (other house-keeping genes showed similar results). The following primers were used: RT KLF4-1 GGCTGATGGGCAAGTTTGTGC, KLF4-2 CTGATGACCGAAGGGCTGGTG, and RT PPIA-1 TTTGGGAAGGTGAAA GAAGG, PPIA-2 ACAGAAGGAATGGTTTGATGG.

Whole-Cell Recordings

Patch-clamp experiments were performed on mRFP-βactin-transfected cultured hippocampal neurons with PBS (vehicle control) or 1 pM Nle 1 -AngIV pretreatment. Recordings were made on DIV12 to DIV14. The culture medium was exchanged by an extracellular solution containing 140 mM NaCl, 2.5mM KCl, 1 mM MgCl 2 , 3 mM CaCl 2 , 25 mM glucose, and 5 mM HEPES; pH was adjusted to 7.3 with KOH, and osmolality was adjusted to 310 mOsM. Cultures were allowed to equilibrate in a recording chamber mounted on an inverted microscope (IX-71; Olympus Optical).
for 30 min before recording. Transfected cells were visualized with fluorescence (Olympus Optical). Recording pipettes were pulled (P-97 Flaming/Brown micropipette puller; Sutter Instrument Company, Novato, CA) from standard-wall borosilicate glass without filament (o.d. = 1.5 mm; Sutter Instrument Company). The pipette-to-bath d.c. resistance of patch electrodes ranged from 4.0 to 5.2 MΩ, and they were filled with an internal solution of the following composition: 25 mM CsCl, 100 mM CsCH3O3S, 10 mM phospho-creatine, 0.4 mM EGTA, 10 mM HEPES, 2 mM MgCl2, 0.4 mM Mg-ATP, and 0.04 mM Na-GTP; pH was adjusted to 7.2 with CsOH, and osmolality was adjusted to 296 to 300 mOsM. Miniature EPSCs (mEPSCs) were isolated pharmacologically by blocking GABA receptor chloride channels with picrotoxin (100 µM; Sigma-Aldrich), blocking glycine receptors with strychnine (1 µM; Sigma-Aldrich), and blocking action potential generation with tetrodotoxin (500 nM; Tocris Bioscience, Ellisville, MO). Recordings were obtained using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Analog signals were low-pass Bessel-filtered at 2 kHz, digitized at 10kHz through a Digidata 1440A interface (Molecular Devices), and stored in a computer using Clampex 10.2 software (Molecular Devices). The membrane potential was held at -70 mV at room temperature (25°C) during a period of 0.5 to 2 h after removal of the culture from the incubator. Liquid junction potentials were not corrected. Data analysis was performed using Clampfit 10.2 software (Molecular Devices) and MiniAnalysis 6.0 software (Synaptosoft, Decatur, GA). The criteria for a successful recording included an electrical resistance of the seal between the outside surface of the recording pipette and the attached cell >2 GΩ and neuron input resistance >240 MΩ. The mEPSCs had a 5-min recording time.
**Statistical Analyses**

Spine density data was analyzed using Kruskal-Wallis non-parametric ANOVA followed by either Dunn’s post-hoc analysis. Electrophysiology data was analyzed using ANOVA followed by Newman-Keuls post-hoc analysis. Numerical data are expressed as means ±S.E.M.

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References


GENERAL CONCLUSIONS

The work presented here is a systematic exploration of leptin signaling required for spine formation in the hippocampus. Most of what we know about leptin signaling has either been shown in heterologous systems or in the hypothalamus. Here, we focus on the hippocampus and identify some distinct molecular cascades initiated by leptin that have not been reported previously. In fact, increase in phosphorylated STAT3 expression a hallmark of leptin signaling in the hypothalamus was not observed in our hippocampal cultures (see Figure4A, chapter2). Leptin activates different transcription factors i.e. CREB and KLF4 in the hippocampus for the purpose of spine formation. It is possible that leptin activates these signaling cascades in the hypothalamus as well. Leptin has been shown to increase excitatory synapse number on the POMC neurons of the hypothalamus (Pinto, Roseberry et al. 2004). It will be interesting to determine the role of the molecular mechanisms described in this dissertation in leptin-induced increase in excitatory synapses in the hypothalamus.

From chapter1, we can conclude that leptin rapidly affects hippocampal neuronal morphology by increasing the number of dendritic filopodia. The signaling mechanism involves the activation of CaMK pathway by leptin that increases TrpC membrane trafficking via β-Pix/Rac1. This study is the first report of leptin activating the CaMK pathway, however, the exact signaling event causing this activation is still unknown. The CaMK pathway can be activated by either intracellular or extracellular calcium stores. In the future, the mechanism by which leptin can increase cytoplasmic calcium levels should be explored. Furthermore,
other neurotrophic factors that also require TrpC trafficking for inducing spine formation, such as BDNF, may act through a similar signaling mechanism to the one described in this report (Amaral and Pozzo-Miller 2007). Although it is clear that some neurotrophic factors require TrpC channels for spine formation, but the exact role of these channels in spine formation is not completely understood. The TrpC channel trafficked in response to leptin passes a mostly sodium current ruling out signaling molecules activated by calcium as mediators of TrpC channel’s effect on spine formation. Nevertheless, sodium current may create a permissive environment for spine formation by shifting the membrane properties of the neuron toward a more depolarized state. This is an important question to address in the future.

Moving on from acute effects of leptin on hippocampal neurons, I focused on chronic leptin effects and excitatory synapse formation in chapter 2. Using different techniques, we were able to show that leptin increases excitatory synapses in cultured hippocampal neurons. The mechanism involved requires CREB-dependent transcription of miR132. Although we did find a reduction in basal spine density in the CA1/CA3 regions of the hippocampus of leptin receptor lacking db/db mice, it will be important to determine if leptin can increase spine formation in normal animals in vivo. Additionally, the question of whether leptin-induced spine formation is required for its effects on memory formation and emotional regulation needs to be directly addressed in the future. If the results from the organotypic slice cultures made from normal rats are any indication then leptin should increase spine formation in vivo as well. By blocking some components of the signaling cascade we describe in this study in vivo (using knockouts, in utero electroporation
Finally in chapter 3, I introduce KLF4, a transcription factor studied extensively in stem cell research and investigate its requirement for leptin-induced synapse formation. From this report, we can conclude that KLF4 is a transcription factor that can regulate synapse formation, especially in response to neurotrophic factors. However, the signaling mechanism involved in KLF4 activation by leptin and its downstream gene targets are still unknown. In the future, the role of KLF4 in synapse formation and its interaction with other transcription factors that can also regulate synapse formation should be explored.

In summary, I present three different molecular mechanisms that mediate leptin’s effects on synapse formation in cultured hippocampal neurons. The ultimate quest will be to understand the interplay between these pathways in an intact animal under normal and pathological conditions. Activation of many different pathways; such as leptin activation of CREB-signaling as well as potentially KLF4-signaling, that apparently lead to the same end results i.e. synapse formation could indicate some redundancy in the system or it could just be a sign of the complex nature of a synapse wherein many different pathways result in subtle changes, all needed for proper synaptic functioning.
References
