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Leptin stimulates synaptogenesis in hippocampal neurons via KLF4 and SOCS3 inhibition of STAT3 signaling

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Abstract

Normal development of neuronal connections in the hippocampus requires neurotrophic signals, including the cytokine leptin. During neonatal development, leptin induces formation and maturation of dendritic spines, the main sites of glutamatergic synapses in the hippocampal neurons. However, the molecular mechanisms for leptin-induced synaptogenesis are not entirely understood. In this study, we reveal two novel targets of leptin in developing hippocampal neurons and address their role in synaptogenesis. First target is Kruppel-Like Factor 4 (KLF4), which we identified using a genome-wide target analysis strategy. We show that leptin upregulates KLF4 in hippocampal neurons and that leptin signaling is important for KLF4 expression *in vivo*. Furthermore, KLF4 is required for leptin-induced synaptogenesis, as shKLF4 blocks and upregulation of KLF4 phenocopies it. We go on to show that KLF4 requires its signal transducer and activator of transcription 3 (STAT3) binding site and thus potentially blocks STAT3 activity to induce synaptogenesis. Second, we show that leptin increases the expression of suppressor of cytokine signaling 3 (SOCS3), another well-known inhibitor of STAT3, in developing hippocampal neurons. SOCS3 is also required for leptin-induced synaptogenesis and sufficient to stimulate it alone. Finally, we show that constitutively active STAT3 blocks the effects of leptin on spine formation, while the targeted knockdown of STAT3 is sufficient to induce it. Overall, our data demonstrate that leptin increases the expression of both KLF4 and SOCS3, inhibiting the activity of STAT3 in the hippocampal neurons and resulting in the enhancement of glutamatergic synaptogenesis during neonatal development.

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Author contributions: GSS, MD, HS, BDW, TJL, SI and GAW conducted the experiments, and analyzed the results. CD prepared the dissociated and organotypic hippocampal cultures. GSS wrote the initial draft of the manuscript. GAW and SMA assisted in the design of the study and writing the manuscript. MZ and SMA conducted and analyzed the results of electrophysiological recording experiments. All authors reviewed the results and approved the final version of the manuscript.

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Keywords

Leptin; STAT3; KLF4; SOCS3; dendritic spine; hippocampus

Introduction

The hormone leptin is a 16 kDa adipocytokine, produced primarily from white adipose tissue (Pan & Myers, 2018). In adult humans and rodents, leptin levels are proportional to body fat mass and signal the repletion of long-term energy stores (Flak & Myers, 2016; Myers, 2004). Interestingly, leptin levels are elevated independent of fat mass at comparable times during development in both rodents and humans (West, 1993). In rodents, leptin surges during post-natal day 7–14 (Ahima, Prabakaran, & Flier, 1998), while in humans, it increases gradually *in utero*, peaking late in the third trimester (Cetin et al., 2000; Sivan et al., 1998). Leptin receptors are widely expressed in the CA1/CA3 and dentate gyrus regions of the hippocampus during this period (Bouret, Draper, & Simerly, 2004; Harvey, 2013), a period of rapid glutamatergic synapse formation. Indeed, mice lacking either leptin (*ob/ob*) or its receptor (*db/db*) have fewer dendritic spines, the main site of glutamate synapses, and exhibit cognitive defects and depressive-like behaviors (Dhar et al., 2014; Sharma, Elased, Garrett, & Lucot, 2010; Yamada et al., 2011), behaviors associated with changes in spine number. Taken together, leptin is an essential neurotrophic signal for hippocampal development.

We have previously demonstrated that leptin is a potent activator of transcription through activating cAMP response element-binding (CREB) protein, and this is required for leptin stimulated synapse formation (Bland, Sahin, et al., 2019; Dhar et al., 2014). CREB regulates the expression of thousands of different genes in neurons, including ones that modulate synaptic development (Dhar et al., 2014; Lesiak et al., 2013). Using ChIP-Seq, we identified ≈ 2000 target genes of CREB, including KLF4 and SOCS3 (Lesiak et al., 2013). Moreover, using high throughput RNA sequencing, we identified KLF4 and SOCS3 to be among the top genes whose transcription is upregulated by leptin (unpublished data).

Kruppel-like factor 4 (KLF4) is a zinc-finger transcription factor that can activate or repress transcription depending on the cell type (Foster et al., 2000; Huang et al., 2005; Xu et al., 2008; Zhao et al., 2004). In the central nervous system (CNS), in retinal ganglion cells (RGCs), expression of KLF4 is regulated developmentally (Moore et al., 2009). Constitutive expression of KLF4 blocks neuronal stem cells from developing into mature neurons (Qin, Liu, Niu, & Zhang, 2011; Qin & Zhang, 2012). KLF4 also blocks axon regeneration post-injury, while its absence enhances axon regeneration through the STAT3-dependent pathway in RGCs (Moore et al., 2009; Qin, Zou, & Zhang, 2013). However, overall, the role of KLF4 in neuronal development is poorly understood (Cheng, Zou, & Cui, 2018; Moore, Apara, & Goldberg, 2011), and its role in regulating hippocampal synaptic development has never been studied.

STAT3 is one of the main downstream signaling proteins that is activated by leptin in the hypothalamus, and other brain regions (Flak & Myers, 2016), and phosphorylated STAT3 (pSTAT3) is often used as a marker for a leptin responsive cell. In contrast, in the

hippocampus, leptin does not activate STAT3 (Caron, Sachot, Prevot, & Bouret, 2010; Dhar et al., 2014; Walker, Long, Williams, & Richard, 2007). Leptin also activates suppressor of cytokine signaling 3 (SOCS3) in the hypothalamus, which then feeds back to reduce leptin signaling (Pan & Myers, 2018). Knockdown of SOCS3 in primary sensory neurons and in RGCs increases the activity of STAT3, which in turn stimulates neurite outgrowth (Miao et al., 2006; Smith et al., 2009). While the interaction of leptin, SOCS3, and STAT3 are well characterized in the hypothalamus (Buettner et al., 2006; Kievit et al., 2006; Mori et al., 2004), little is known how they interact in the hippocampus or the roles of SOCS3 and STAT3 in leptin's effects on synaptogenesis.

In this study, we show that leptin increases expression of KLF4 and SOCS3, both of which in turn inhibits STAT3 and increase synaptogenesis. Targeted knockdown of either KLF4 or SOCS3 and expression of constitutively active STAT3 blocks the effects of leptin. This study identifies a novel mechanism by which leptin regulates synaptogenesis in the hippocampal neurons.

Results

Leptin stimulates the expression of KLF4 in hippocampal neurons

We performed whole transcriptome sequencing (RNA-seq) analysis after leptin treatment in day *in vitro* (DIV) 7 cultured hippocampal neurons and identified KLF4 as one of the most inducible genes by leptin (data not shown). To confirm the RNA-seq data, we did quantitative PCR (qRT-PCR) following a time course of leptin treatment (50nM). We found that mRNA levels of KLF4 increased to 12.72-fold of vehicle treated neurons after 1 hour of leptin treatment, $p<0.001$ (normalized to housekeeping gene PPIA). This increase sustained for 2 hours and then dropped to control levels at 4 hours and beyond, suggesting a transient regulation of KLF4 transcription by leptin (0.5 hour: 249 ± 13%, 1 hour: 1272 ± 70% $p<0.001$, 2 hours: 1167 ± 64% $p<0.001$, 4 hours: 232 ± 12%, 8 hours: 205 ± 11%) (Figure 1A). Our previous study that included a chromatin immunoprecipitation followed by sequencing (ChIP-Seq) for CREB done with P1 rat hippocampi identified that the KLF4 promoter region is targeted by CREB with cumulated ChIP-Seq tags of 28 (Lesiak et al., 2013). To verify our ChIP-Seq analysis, we did CREB-ChIP and IgG-ChIP on P10 rat hippocampi followed by qPCR, targeting KLF4 promoter (% of input: CREB-ChIP: 0.37 ± 0.04%, IgG-ChIP: $0.005 \pm 9e^{-5}$%, $p<0.001$). We have previously shown that leptin can activate the MEK/ERK-CREB signaling cascade in hippocampal neurons (Lesiak et al., 2013). To test if MEK/ERK-CREB signaling cascade is involved in this case, we used U0126 (20μM) an inhibitor of the MEK/ERK-CREB pathway (Buettner et al., 2006; Dhar et al., 2014), and analyzed expression levels of KLF4 protein by western blot in primary hippocampal cultures. Expression of KLF4 increased to 1.53 ± 0.25-fold after 2 hours of leptin treatment, $p=0.017$, and this increase was blocked by U0126 (Figure 1B and 1C).

Next, we determined if leptin regulates expression of KLF4 *in vivo*. In rodents, leptin levels peak at around postnatal day (PND)10 (Ahima et al., 1998). Interestingly, in situ hybridization for KLF4 mRNA shows that the expression of KLF4 increases gradually during hippocampal development; while the expression is very low at PND4 (prior to leptin surge), it is detectable at PND14 in the CA1 and dentate gyrus, and it is present in all

the hippocampal regions at PND28. Interestingly, at PND56, the expression of KLF4 is almost undetectable in the hippocampus, suggesting that its expression is essential during development more than in adulthood (Allen Developing Mouse Brain Atlas 2008). We asked whether the protein levels show the same pattern of change as its mRNA levels and western blot analysis showed that the expression of KLF4 protein increases developmentally in wild-type animals. At PND10, KLF4 had increased to 193 ± 19%, $p<0.001$, and at PND20, it had continued to increase to 336 ± 18%, $p=0.007$ (fold change and p-values are expressed compared to PND5) (Figure 2A). Interestingly, the expression of KLF4 was lower in mice that lack leptin synthesis (*ob/ob*) (56 ± 13% of wild-type, $p=0.025$) (Figure 2B). Taken together, these data support the hypothesis that KLF4 expression is regulated in the developing hippocampus, and this requires the presence of leptin.

KLF4 stimulates glutamatergic synaptogenesis and is required for leptin-induced synaptogenesis

Since both leptin and accordingly KLF4 levels increase when hippocampal synaptogenesis is at its peak and leptin is already known to increase hippocampal synaptogenesis, we wanted to address the role of KLF4 in leptin-induced synaptogenesis. In the hippocampus, excitatory synapses are located on a distinct morphological structure called spines that are small protrusions with actin-enriched spherical heads connected to a dendrite via a thin neck. They are commonly classified into three subcategories: stubby, mushroom, and filopodia, and the first two types of these are considered to be mature spines, where functional glutamatergic synapses are mainly located (Bourne & Harris, 2007).

To address the role of KLF4 in leptin-induced spine formation, we first verified the expression of the wtKLF4 construct and the efficiency of its targeted knockdown in HEK293T cells (expression level of the protein was reduced to 48% in the presence of shRNA, Figure 3A). Primary neurons were transfected with either KLF4 or shKLF4 constructs, along with Clover tagged β-actin expressing vector as the cell fill on DIV5–6, stimulated with vehicle or leptin on DIV7 (in case of shKLF4 experiment) and analyzed for spine density on DIV11–12. Overexpression of KLF4 significantly increased both stubby (135 ± 6.7% of control, $p=0.0014$) and mushroom (197 ± 14 % of control, $p<0.001$) type mature spine density as leptin stimulation (stubby: 182 ± 11% of control, $p<0.001$ and mushroom: 159 ± 9.6% of control, $p=0.011$), while shKLF4 expression blocked the leptin-induced spinogenesis (Figure 3B and C). However, filopodia type spines, considered to be immature ones, did not significantly change among different treatment groups ($H(4)=14.62$, all p-values>0.05). To verify that stubby and mushroom type mature spines represent functional synapses with corresponding presynaptic sites, we analyzed colocalization of spines with vGlut1, a marker for glutamatergic presynaptic boutons. We found that in control neurons, the percentage of colocalization between mature spines and vGlut1 was 90.8 ± 1.4%; in leptin-treated neurons, it was 91.9 ± 2.1%; in wtKLF4-transfected neurons, it was 93.4 ± 1.15%; and finally, in shKLF4-transfected neurons it was 89.1 ± 2.1%, suggesting that almost all of the scored mature spines represent functional glutamatergic synaptic sites and the percentage of spines with presynaptic markers were not significantly different across different conditions (Bland, Zhu, et al., 2019; Dhar et al., 2014).

To further confirm that leptin and KLF4-induced spines represent a change in the number of functional synapses, we performed whole-cell patch clamp experiments on DIV11–12 hippocampal neurons and recorded mini excitatory post synaptic currents (mEPSCs), which are transfected and treated the same way as in spine analysis. The mEPSCs frequency in control neurons was 2.32 ± 0.25 Hz, and with leptin treatment, the frequency of mEPSCs increased to 6.39 ± 0.41 Hz, $p<0.001$, and with KLF4 overexpression, it increased to 4.90 ± 0.42 Hz, $p<0.001$. Furthermore, targeted knockdown of KLF4 expression blocked the leptin-induced increase in frequency of mEPSCs (shKLF4 = 1.46 ± 0.21 Hz; shKLF4+leptin = 1.44 ± 0.18 Hz) ($H(3)=43.95$, control vs. shKLF4 and vs. shKLF4+leptin >0.05, leptin vs. shKLF4+leptin $p<0.001$) (Figure 3D–F). In contrast, both amplitude and decay time of mEPSCs were not affected by either leptin treatment, KLF4 overexpression or targeted knockdown of KLF4 (amplitude (-pA): control = 20.1 ± 0.8, leptin = 20.8 ± 1.1, shKLF4 = 19.3 ± 1.2, shKLF4+leptin = 19.7 ± 1.1, wtKLF4 = 18.9 ± 0.6 and decay time (ms): control = 2.55 ± 0.13, leptin = 2.99 ± 0.15, shKLF4 = 3.26 ± 0.23, shKLF4+leptin = 3.23 ± 0.19, wtKLF4 = 2.36 ± 0.12). Together with the increase in number of morphological spines and colocalization with the presynaptic marker (vGlut1), these data support the hypothesis that KLF4 is required for leptin-induced glutamatergic synapse formation and its expression alone is sufficient to increase synapse formation (Figure 3D–F).

We further confirmed the role of KLF4 using rat organotypic slice cultures as they maintain more of the normal hippocampal circuits (Bahr, 1995; Caeser & Aertsen, 1991). We delivered the DNA constructs biolistically (Wayman et al., 2006). While leptin increased both stubby and mushroom type mature spine density (stubby spines: 233 ± 24%, $p=0.006$, mushroom spines: 160 ± 14%, $p=0.043$), shKLF4 blocked the effects of leptin, suggesting that KLF4 is also required in leptin-induced synaptogenesis in a more conserved hippocampal circuit model (Figure 3G). Note that, in organotypic slice preparations, with shKLF4, we observed a significant decrease in both type of mature spine density compared to control conditions (stubby spines: 67 ± 18%, $p=0.041$, mushroom spines: 50 ± 11%, $p=0.031$), suggesting that KLF4 expression is required for the process of spine maturation. Overall, these data support the conclusion from the dissociated culture preparations that shKLF4 is required for the leptin-induced formation of mature spines in hippocampal neurons.

KLF4 requires its pSTAT3-binding domain to induce spinogenesis

KLF4 can produce its effects by binding directly to the active form of STAT3, pSTAT3 (Qin et al., 2013), or by acting as a transcription factor (Ghaleb & Yang, 2017; Segre, Bauer, & Fuchs, 1999). To determine the contribution of each in spinogenesis, we used three constructs of KLF4; first, N400, in which both transcription activity and DNA binding domains were deleted; second, N454, in which KLF4 transactivation domain was deleted, thus rendering it unable to recruit transcriptional machinery; and finally, N300, in which STAT3 binding domain was deleted (Qin et al., 2013) (Figure 4A). We observed that neither DNA binding domain nor transcription activity of KLF4 was required to induce spinogenesis in hippocampal neurons (stubby spines: KLF4-N400 = 130 ± 5.8% $p=0.002$, KLF4-N454 = 148 ± 10% $p<0.001$, mushroom spines: KLF4-N400 = 155 ± 10% $p<0.001$, KLF4-N454 = 187 ± 20% $p<0.001$) (Figure 4B). On the other hand, the deletion of the

STAT3 binding site of KLF4, N300, was not able to induce spinogenesis, suggesting that KLF4 increases spine formation by binding and inhibiting STAT3 activity. From these experiments, we conclude that KLF4 increases spinogenesis by its inhibitory interaction with STAT3, rather than acting as a transcription factor.

SOCS3 stimulates glutamatergic synaptogenesis and is required for leptin-induced synaptogenesis

SOCS3 is a well-known inhibitor of LepRb activation of STAT3 in the hypothalamus, providing negative feedback on leptin signaling (Bjorbak et al., 2000). Since our findings suggest that KLF4-induced spine formation requires the pSTAT3 binding domain, we addressed the role of SOCS3 in mature spine formation in hippocampal neurons. First, we showed that expression of SOCS3 increases as early as 30 minutes after leptin stimulation and stays up more than 2 hours, suggesting that SOCS3 is an early response gene in the hippocampal neurons, as it is in the hypothalamus (Elias et al., 1999) (15 min.; 150 ± 50%, 30 min.; 227 ± 30%, $p<0.001$, 60 min.; 213 ± 32%, $p<0.001$ and, 120 min.; 186 ± 27%, $p=0.005$) (Figure 5A and 5B). In the hypothalamus, leptin increases SOCS3 expression by activating STAT3, which activates the transcription of SOCS3. However, in the hippocampus, leptin does not increase the activity of STAT3 (Dhar et al., 2014). Based on our ChIP-seq study, SOCS3 is a direct target of the CREB transcription factor with a cumulated ChIP-seq peak of 20 (Lesiak et al., 2013). We verified this, by performing ChIP followed by qPCR targeting SOCS3 promoter region (% of input: CREB-ChIP: 0.022 ± 0.004, IgG-ChIP: $0.004 \pm 8.8e^{-4}$, p-value=0.02). Furthermore, we found that inhibition of MEK/ERK signaling pathway, through which leptin activates CREB (Lesiak et al., 2013), blocks the leptin-induced increase of SOCS3 expression (2 hours; 160 ± 9%, p-value=0.008; U0126+leptin; 102 ± 14%, p-value>0.05. In this experiment, we used the same hippocampal cultures with the experiment reported in Figure 1D; hence the same β-actin blots were shown in both figures) (Figure 5C and 5D).

Next, we asked if SOCS3 overexpression is sufficient to increase synaptogenesis in hippocampal neurons by itself, and whether targeted knockdown of SOCS3 inhibits leptin-induced synaptogenesis. Expression level of the protein was reduced to 45% in the presence of shSOCS3, while importantly there was no difference in endogenous SOCS3 levels between only pCAGGS-Clover transfected and non-transfected neurons (fold change over pCAGGS-Clover; 109%) (Figure 5E). We showed that both stubby (146 ± 10% of control, $p<0.001$) and mushroom (168 ± 14% of control, $p<0.001$) type spines significantly increased with overexpression of SOCS3. Interestingly, addition of leptin onto the SOCS3 transfected neurons did not further increase the glutamatergic spinogenesis (stubby: 157 ± 7.8% $p<0.001$, mushroom: 199 ± 10.6% $p<0.001$) suggesting that they use the same mechanism of action and/or spine numbers are tightly regulated during development (reviewed in (Wefelmeyer, Puhl, & Burrone, 2016)). Furthermore, knockdown of SOCS3 blocked the leptin-induced increase in spine number (Figure 5F and 5G). When we looked at the whole-cell recordings, overexpression of SOCS3 alone was sufficient to increase the frequency of mEPSCs and leptin treatment did not further increase the frequency, same as with the spines. In contrast, shSOCS3 blocked the effects of leptin (control = 1.15 ± 0.14 Hz; leptin = 4.77 ± 0.34 Hz; SOCS3 = 4.03 ± 0.43 Hz; SOCS3+leptin = 4.69 ± 0.62 Hz,

shSOCS3 = 1.57 ± 0.12 Hz; shSOCS3+leptin = 2.14 ± 0.35 Hz) ($H(5)=76.79$, control vs. leptin $p<0.001$, control vs. SOCS3 $p<0.001$, control vs. SOCS3+leptin $p<0.001$, control vs. shSOCS3 $p=0.38$, leptin vs. shSOCS3+leptin $p<0.001$) (Figure 5H–J). However, similar to KLF4, the amplitude of mEPSCs was not affected by either SOCS3 overexpression or targeted knockdown of SOCS3 (amplitude (-pA): control = 25.1 ± 1.5, leptin = 24.6 ± 0.6, SOCS3 = 23.9 ± 1.2, SOCS3+leptin = 25.8 ± 1.5, shSOCS3 = 25.2 ± 0.5, shSOCS3+leptin = 28.6 ± 1.04 and decay time (ms): control = 2.79 ± 0.13, leptin = 2.33 ± 0.06, SOCS3 = 2.29 ± 0.07, SOCS3+leptin = 2.30 ± 0.09, shSOCS3 = 2.49 ± 0.08, shSOCS3+leptin = 2.50 ± 0.13). These data support our hypothesis that SCOS3 is both required for leptin-induced glutamatergic synapse formation and that its expression alone is sufficient to increase synapse formation.

Suppression of STAT3 activity increases spinogenesis

Both KLF4 and SOCS3 have previously been shown to inhibit the activity of STAT3 (Bjorbak et al., 2000; Estrada et al., 2018; Park, Lin, Benveniste, & Lee, 2016; Qin et al., 2013). Since the effects of leptin on glutamatergic synaptogenesis require both KLF4 and SOCS3, we wanted to test the hypothesis that leptin, through upregulation of KLF4 and SOCS3, inhibits STAT3 activity in the hippocampal neurons. We quantified mean pSTAT3 fluorescence intensity both in the nucleus and whole neuron (soma and proximal dendritic branches) following treatment with leptin or vehicle for 2 hours. Our data showed that leptin decreased the pSTAT3 levels significantly in both nucleus (61 ± 4.2% of control, $p=0.002$) and whole neuron (69 ± 4.0% of control, $p=0.007$). Furthermore, while overexpression of either KLF4 or SOCS3 was enough to decrease pSTAT3 levels, knockdown of either KLF4 or SOCS3 blocked the leptin-induced decrease in pSTAT3 levels both in nucleus (wtKLF4 = 60.8 ± 4.9% $p<0.001$, wtKLF4+Leptin = 61.3 ± 5.0% $p=0.01$, shKLF4 = 88.1 ± 4.4%, shKLF4+Leptin = 85.6 ± 4.0%, SOCS3 = 51.7 ± 4.6% $p<0.001$, SOCS3+Leptin = 55.7 ± 5.5% $p<0.001$, shSOCS3 = 94.1 ± 7.3%, shSOCS3+Leptin = 88.6 ± 5.7%) and whole neuron (wtKLF4 = 62.7 ± 4.1% $p<0.001$, wtKLF4+Leptin = 62.6 ± 4.3% $p=0.01$, shKLF4 = 93.8 ± 5.4%, shKLF4+Leptin = 79.1 ± 3.5%, SOCS3 = 55.6 ± 4.0% $p<0.001$, SOCS3+Leptin = 55.0 ± 5.2% $p<0.001$, shSOCS3 = 85.2 ± 4.3%, shSOCS3+Leptin = 76.1 ± 5.0%) (Figure 6A and 6B).

To directly demonstrate the role of STAT3 in glutamatergic spine formation, we used a constitutively active form of STAT3 (A661C and N664C) (Takahashi & Yamanaka, 2006). We hypothesized that if leptin blocks the activity of STAT3 via KLF4 and SOCS3 to increase mature spine formation, then the expression of caSTAT3 should block the effects of leptin. We quantified the spines after overexpressing the caSTAT3 and treating with leptin or vehicle. While caSTAT3 alone did not affect basal spine numbers, leptin effects were blocked in the presence of caSTAT3, suggesting that the inhibition of STAT3 activity is required for the leptin-induced mature spine formation (stubby spines: caSTAT3 = 112 ± 7.1%, control vs. caSTAT3 $p=0.92$, caSTAT3+leptin = 113 ± 7.2%, leptin vs. caSTAT3+leptin $p<0.001$; mushroom spines: caSTAT3 = 103 ± 11%, control vs. caSTAT3 $p=0.43$, caSTAT3+leptin = 92 ± 11%, leptin vs. caSTAT3+leptin $p<0.001$) (Figure 6C and 6D).

To verify the role of STAT3 in spinogenesis, we also used shSTAT3 to knockdown the expression of STAT3 (expression of the protein was reduced to 40% in the presence of shRNA, Figure 6E), and dominant-negative (dn) STAT3 to block its activity (Wen & Darnell, 1997). We showed that both shSTAT3 and dnSTAT3 increased the number of mature spine types; stubby (shSTAT3 = 163 ± 6.8% $p<0.001$, dnSTAT3 = 179 ± 11% $p<0.001$) and mushroom (shSTAT3 = 196 ± 11% $p<0.001$, dnSTAT3 = 220 ± 17% $p<0.001$), similar to leptin treatment condition. As a negative control, we used scrambled shRNA (Dhar et al., 2014), which does not have any specific target, and showed that it did not change spine formations (Figure 6F). This data supports our hypothesis that STAT3 suppresses spine formation, as decreasing its expression and inhibiting its activity increases it to similar levels with leptin treatment.

Discussion

Leptin is a type 1 adipocytokine that is best known as a feedback signal for the metabolic state of the individual to the central nervous system (Allison & Myers, 2014; Elmquist, Maratos-Flier, Saper, & Flier, 1998; Zhang et al., 1994). However, over the last decade, it has also been identified as a crucial neurotrophic factor for normal CNS development, including hippocampal development (Bouret, 2010; Caron et al., 2010). While its actions to regulate food intake have been widely studied (Badman & Flier, 2005; Friedman, 2014; Pan & Myers, 2018), we are now starting to understand the molecular pathways by which leptin controls synapse formation (Bland, Zhu, et al., 2019). Here, we identified two novel proteins required for leptin-induced glutamatergic synaptogenesis in hippocampal neurons, namely, KLF4 and SOCS3 (Figure 7). To our knowledge, this is the first study that identified the role of both of these proteins in glutamatergic synaptogenesis in hippocampal neurons. Specifically, we show that 1) expression of both KLF4 and SOCS3 is highly inducible by leptin in hippocampal neurons, 2) KLF4 expression is dependent on leptin signaling *in vivo*, 3) KLF4 and SOCS3 are both required for leptin-induced hippocampal glutamatergic synapse formation and induce spine formation when overexpressed 4) constitutively active STAT3 blocks leptin-induced spine formation, while inhibition of STAT3 increases it.

Leptin induces the expression of KLF4 and SOCS3 in hippocampal neurons.

STAT3 is a hallmark transcription factor induced by leptin in the hypothalamus and midbrain (Buettner et al., 2006) and is routinely used as a measure of neuronal activation by leptin. However, we and others have previously shown that brief leptin application (up to 30 minutes) does not increase STAT3 activation (phospho-STAT3, pSTAT3) in hippocampal neurons (Dhar et al., 2014; Walker et al., 2007). One likely reason for this lack of activation is that pSTAT3 levels are already high in control/unstimulated developing hippocampal neurons (Dhar et al., 2014; Nicolas et al., 2012). Here, for the first time, we demonstrated that longer leptin treatment actually decreases the activity of STAT3, i.e. pSTAT3 levels, in cultured hippocampal neurons. Interestingly, leptin increases pSTAT3 levels in hippocampal progenitor cells, an effect required for neurogenesis, suggesting that leptin regulation of STAT3 is cell type specific even within the same brain region (Garza, Guo, Zhang, & Lu, 2008; H. S. Moon, Dincer, & Mantzoros, 2013). It remains to be established if there are other brain regions, or cell types, in which leptin also does not activate (and potentially even

inhibits) STAT3; but our findings suggest that pSTAT3 may not reveal all leptin-sensitive neurons as most hippocampal neurons would not be identified. This inhibition of STAT3 by leptin in most hippocampal neurons is likely due to the differential regulation of other signaling pathways that interact with STAT3, including potentially KLF4 and SOCS3, as discussed below.

Leptin stimulates other transcription factors in hippocampal neurons, including CREB, which is a key transcription factor that mediates many of leptin's effects in the hippocampus both *in vivo* and *in vitro* (Dhar et al., 2014; Vaisse et al., 1996; Walker et al., 2007). Here we show that CREB is bound to the promoter region of KLF4 in hippocampal neurons, as it is also in hypothalamic AgRP neurons (Nakajima et al., 2016), and that activation of CREB via MEK/ERK pathway, is required for transcriptional upregulation of KLF4 by leptin. We found that KLF4 protein levels *in vivo* increase during the time of the leptin surge (PND10 onwards, (Ahima et al., 1998)), mirroring the changes in KLF4 mRNA, reported by the Allen Brain Institute. Furthermore, KLF4 protein is reduced in mice lacking leptin synthesis (*ob/ob*), suggesting that leptin is one of the critical regulators of KLF4 expression in the hippocampus *in vivo*. KLF4 is induced by leptin in adipose tissue (Gan et al., 2017), an action required for leptin's anti-inflammatory effects. Furthermore, Turner *et al*. reported that leptin may also induce KLF4 in myocardial cells (Turner, Huang, Govindarajan, & Caplice, 2013), suggesting that this is a common signaling pathway for leptin in multiple tissues. Interestingly, however, leptin has been shown to reduce the expression of KLF4 in AgRP neurons in the arcuate nucleus of the hypothalamus, a mechanism thought to be involved in leptin's inhibition of food intake (Imbernon et al., 2014). This suppression of KLF4 by leptin is through the activation of PI3 kinase and STAT3. However, since leptin does not activate STAT3 in the hippocampus, this may underlie the differential regulation of KLF4 expression.

We also found that leptin upregulates SOCS3, and this occurs earlier than KLF4 upregulation, suggesting that SOCS3 is an early response gene of leptin signaling in the hippocampus, similar to in the hypothalamus (Flak & Myers, 2016). However, while in the hypothalamus SOCS3 expression is induced by STAT3, in the hippocampus, we showed that leptin increases SOCS3 expression by CREB-dependent transcription through activation of the MEK/ERK pathway. SOCS3 is induced by other cytokines and growth factors (Carow & Rottenberg, 2014; Shi, Tzameli, Bjorbaek, & Flier, 2004) and indeed is induced by CREB in hypothalamic cells and microglia (Chakrabarti, Jana, Roy, & Pahan, 2018; Kageyama, Hanada, Iwasaki, & Suda, 2009; Yang & McKnight, 2015).

KLF4, SOCS3 and suppression of STAT3 signaling are required for leptin-induced spine formation.

The majority of excitatory synapses are established on the dendritic spines, and these compartmentalized structures are highly dynamic and affected by on-going activity such that the number and shape of spines are one of the first indicators of continuous and stabilized activity (reviewed in (Hering & Sheng, 2001)). On the other hand, the lack of mature spines is associated with deficits in cognition as well as depression and anxiety (Penzes, Cahill, Jones, VanLeeuwen, & Woolfrey, 2011; Pittenger & Duman, 2008). We show that both

KLF4 and SOCS3 induce the formation of mature spines with functional glutamatergic synapses rather than immature filopodia, supporting a role for both KLF4 and SOCS3 in normal hippocampal function.

KLF4 and SOCS3 both block axon regeneration in retinal ganglion cells by inhibiting STAT3 activity (Qin et al., 2013; Smith et al., 2009). While the overexpression of either KLF4 or SOCS3 was enough to induce spinogenesis, the presence of constitutively active STAT3 (caSTAT3) blocked the effects of leptin, presumably due to being resistant to inhibition by either KLF4 or SOCS3. Interestingly, caSTAT3 did not further decrease the basal spine density, suggesting that either already existing pSTAT3 levels did not change significantly or how much active STAT3 can block normal development of spines is tightly regulated due to homeostatic synaptic plasticity (Turrigiano, 2012). Since high levels of pSTAT3 are present in hippocampal neurons (Dhar et al., 2014; Nicolas et al., 2012), STAT3 might be in its maximal effective concentration, and the addition of more active STAT3 does not affect basal spinogenesis; however, its inactivation is required for leptin-induced spine formation. Intriguingly, leptin induces synaptogenesis in hypothalamic neurons (Horvath, 2006), while also inducing STAT3, again suggesting that STAT3 plays a different role in hypothalamic and hippocampal neurons.

KLF4 and SOCS3 might have other effects during the leptin surge in addition to blocking the activity of STAT3. Indeed, a recent study addressing the role of KLF4 in neurogenesis in cortical neurons identified RNA-binding protein Staufen 1 (Stau1) as an interaction partner of KLF4 (B. S. Moon et al., 2018). Stau1 is a protein of interest because it is a target of CREB based on our genome-wide screen of CREB occupancy (Lesiak et al., 2013), suggesting that it can be induced by leptin. Furthermore, Stau1 is identified to be a positive regulator of excitatory synaptogenesis and long-term potentiation in hippocampal neurons (Krichevsky & Kosik, 2001; Lebeau et al., 2008). It is possible that KLF4 and Stau1 are upregulated upon leptin surge via CREB, and they regulate synaptogenesis coordinately, which should be addressed in future studies.

Physiological implications

We show that constitutively active STAT3 blocks leptin-induced spine formation, suggesting that STAT3 is a negative regulator of spinogenesis. This is consistent with the proposed role of STAT3 in hippocampal long-term depression (LTD) in both juvenile (Nicolas et al., 2012) and adult mice (McGregor, Irving, & Harvey, 2017), as LTD is associated with a decrease in spine number and altered spine shape in the hippocampus (Nagerl, Eberhorn, Cambridge, & Bonhoeffer, 2004; Zhou, Homma, & Poo, 2004). Interestingly, Nicolas *et al.* reported that STAT3 induces NMDA receptor-dependent LTD in the hippocampal neurons without requiring either nuclear localization or its transcription activity (Nicolas et al., 2012). Moreover, a recent study by McGregor *et al.* confirmed that LTD at adult hippocampal synaptic connections involves in JAK-STAT signaling; however, they reported that this LTD requires STAT3-dependent gene transcription at this age (McGregor et al., 2017). It is possible that the late phase of LTD requires transcription activity of STAT3, while the organization of cytoskeleton components by STAT3 underlies the earlier phases (Gao & Bromberg, 2006; Kapitein et al., 2011; Ng et al., 2006). Taken together, STAT3

can be both a transcription factor and allosteric regulator of the cytoskeleton both during development and adulthood (Gao & Bromberg, 2006; Ng, Lim, Lin, Zhang, & Cao, 2009; Ng et al., 2006), but future studies are required to address which role of STAT3 is necessary to suppress spine formation in hippocampal neurons.

Our current study focuses on developmental time points. However, leptin has also been reported to have critical effects on synaptic plasticity, including bi-directional effects on long-term plasticity. For example, acutely leptin has been shown to induce LTP and LTD by modulating Schaffer collateral-CA1 synapses and Temporoammonic-CA1 synapses, respectively (McGregor, Clements, Farah, Irving, & Harvey, 2018; Moult & Harvey, 2011). The chronic effects of leptin on hippocampal synaptic plasticity and whether the molecular pathways we have elucidated here play a role in the adult remains to be established. In addition, both CA3 and DG regions contain leptin-responsive neurons (Dumon et al., 2018; Wang, Zhang, & Lu, 2015), and identifying the effects of leptin in these neurons will also provide a better understanding of how leptin signaling may facilitate the integration of an input through hippocampal circuitry and consequently adult hippocampal functioning.

In summary, our study describes novel molecular mechanisms by which leptin induces synaptogenesis in the developing hippocampus. We showed that leptin upregulates expression of both KLF4 and SOCS3 by the CREB transcription factor, which in turn increases synaptogenesis in hippocampal neurons. Moreover, we found that STAT3 is a negative regulator of spine formation and its inhibition, including by leptin-induced upregulation of KLF4 and SOCS3, induces spine formation.

Materials and Methods

Drugs and DNA constructs.

Constructs expressing tagged proteins were constructed by amplifying KLF4 and SOCS3 from rat cDNA and cloned into pCAGGS destination vectors containing the designated tag using Gateway Cloning (ThermoFisher). The short hairpin RNAs (shRNA) targeting the KLF4 (5’-GCCAGAGGAGCCCAAGCCAAAG-3’), SOCS3 (5’-CAGTTAGCCCAGCAGAAGATA-3’) and STAT3 (5’-AGAACGTGAACTTCTTCACTA-3’) was cloned into pSUPER, pLKO.3G and pLKO.TRC vector, respectively. The constructs expressing shLepRb (5’-GCTCACTGTCTGTTCAGTGAC-3’) were used as previously described (Dhar et al., 2014). Different deletions of KLF4 constructs are generated as described previously (Qin et al., 2013). pMXs-Stat3-C was a gift from Shinya Yamanaka (Addgene plasmid #13373; http://n2t.net/addgene: 13373; RRID: Addgene_13373). Stat3 Y705F Flag pRc/CMV was a gift from Jim Darnell (Addgene plasmid # 8709; http://n2t.net/addgene:8709; RRID: Addgene_8709). Full length rat recombinant leptin (50nM, Peprotech #400–21) and U0126 (20μM, Tocris #1144) was used as described in the text and figure legends.

Cell culture.

Animals used for hippocampal cultures was carried out in compliance with Washington State University IACUC approved protocols 03717–019 and 04409–006. Hippocampal

neuronal cultures were prepared from equal numbers of P1 female and male Sprague-Dawley rat pups. Briefly, dissected hippocampi were collected in cold Hibernate A (Brain Bits). Then, the hippocampi were coarsely chopped and collected in the plating media (1% B27, 1% Glutamax, 10% Horse Serum, 2% HEPES, pH 7.5 in Neurobasal A Media) containing 0.25% Papain (Sigma #P3125) and 0.2% DNase (Sigma Aldrich #D5025) and incubated at 37°C for 20–25 minutes with mild shaking. After settling the tissue down, the media containing papain and DNase was removed and warm plating media was added, then the tissue were triturated 6–10 times with fire-polished glass pipette in this media to achieve suspension of single cells. The cells were counted and plated at the density of 3×10^4 cells/cm^2 for 24-well plates to be used for spine and electrophysiology experiments, and 4.7×10^4 cells/cm^2 for 6-well plates to be used for biochemistry experiments, on plates that were coated previously with Poly-L-Lysine. After 2–3 hours of plating, the plating media was changed with growth media (1% B27 and 1% Glutamax in Neurobasal A Media) (Dhar et al., 2014). On *day in vitro* (DIV) 4, the feeding media (1% B27, 1% Glutamax and 5μM cytosine-D-arabinofuranoside (AraC) in Neurobasal A Media) was added to neurons to constitute one third of the total media. Hippocampal neurons were transfected with various constructs on DIV5–6, treated with leptin (50nM) on DIV7–8, and on DIV11–12, cells were either fixed (4% paraformaldehyde in PHEMS buffer [60mM PIPES, 25mM HEPES, 1mM $MgCl_2$, 5mM EGTA], 87.6mM Sucrose, pH 7.4) and mounted with Elvanol (10g polyvinyl alcohol (VWR, #JTU232–8), 1.94g Tris Base, 40mL Glycerine (Sigma Aldrich #G7043), 0.6g DABCO (Sigma Aldrich #2522) to be used for imaging or they were used for electrophysiological recordings. All control conditions received the same amount of media at the time of reagent stimulation.

Hippocampal Slice Culture Preparation & Transfection.

Organotypic hippocampal slices were prepared from post-natal day 5 Sprague-Dawley rats from either sex. Briefly, the hippocampi were dissected in cold, oxygenated low sodium ACSF (1mM $CaCl_2$, 10mM D-Glucose, 4mM KCl, 5mM $MgCl_2$, 26mM $NaHCO_3$, 234mM Sucrose and 0.1% v/v Phenol Red). Excess liquid drained from the hippocampi and they were aligned on Teflon sheet placed on the tissue chopper side by side. The hippocampi were cut into 400μm thick slices. Well defined and undamaged slices were selected under light microscope and placed onto membrane inserts which were placed in the 6-well plates with Slice Culture Media (SCM; 8.4g/L MEM Eagle medium, 20% Horse serum heat inactivated, 1mM L-Glutamine, 1mM $CaCl_2$, 2mM $MgSO_4$, 1mg/l Insulin, 0.00125% Ascorbic Acid solution, 13mM D-Glucose, 5.2mM $NaHCO_3$, 30mM HEPES, adjusted pH to 7.27–7.28, and osmolality to 320mOsm) (Opitz-Araya & Barria, 2011). The slice cultures were maintained for 3 days and 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 24 hours before stimulation with 50nM leptin for 2 days. Slices were fixed, mounted, and imaged using a confocal microscope. Dendritic spine density was measured as described in spine quantification.

Transfection.

Primary hippocampal cultures were transfected with Lipofectamine 2000 (Life Technologies #11668019) on DIV5–6. Native media was collected before transfection and replaced with warm growth media. Lipofectamine 2000 and experimental DNA plasmids (0.5μg/well for 24-well plates, 2μg/well for 6-well plates) were added to cells and incubated for 30min. The media containing Lipofectamine was then aspirated and replaced back with native media. This protocol produces a transfection efficiency of only 3% - 5% of total neurons transfected, which was desirable for spine quantification analysis.

Imaging and Spine Quantification.

Hippocampal neurons were transfected on DIV5–6 with designated DNA constructs (for shRNA constructs, 0.25μg DNA and for overexpression, 0.125μg DNA were used) and Clover-β-actin (0.025μg) to allow for visualization of dendritic spine density and morphology. Confocal fluorescent images were obtained using Metamorph software and a Leica DMI6000 SD confocal microscope equipped with a Yokogawa CSU-X1 spinning disk, SD EMCCD, and a 60× oil immersion lens (NA: 1.4). Z-stacks producing 3μm depth have been acquired for each image and fluorescence channel. Spines from 1–2 dendritic segments, that were 150–200μm away from soma, were imaged from a minimum of 8 neurons from at least three independent hippocampal culture. If more than one dendrite segment were imaged and counted, the average had been considered as one neuron. Spines with a discrete neck with a head of at least twice the diameter of neck were defined as mushroom type; spines with a short or non-existent neck with a head were defined as stubby type; and, spines with a long thin neck with or without a small head with diameter less than twice the diameter of the neck were defined as filopodia type (Dhar et al., 2014).

Chromatin immunoprecipitation followed by quantitative PCR.

ChIP protocol was done as described previously (Bayam et al., 2015). Two postnatal-day 10 rat hippocampi were used for each ChIP experiment. Antibodies used for pull-down were anti-CREB (Cell signaling #9197, 1:100) and anti-IgG (Cell signaling #2729, 1:100). After antibody incubations beads were washed with three wash buffers, twice for 10 minutes. (Low Salt Wash Buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH 8.0), 150mM NaCl; High Salt Wash Buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH 8.0), 500mM NaCl; LiCl Wash Buffer: 1%NP-40, 1%Na-DOC, 1mM EDTA, 10mM Tris-HCl (pH 8.0), 250mM LiCl). After elution of DNA, input and ChIP'ed DNA were each dissolved in 30μl water and 1μl of each was used in a single qPCR reaction. Primers were designed against conserved CpG islands to target promoter regions. KLF4; forward: 5'-CCACCTCTTACCTTGGTGTTAG-3', reverse: 5'-CTGCCATAGGAACTGGAC-3'; SOCS3; forward: 5'-GTCCTTTGCATGATCCAC-3', reverse: 5'-TCCTACCTAGTCCCGAAGCA-3'. As a positive control, qPCR targeting c-fos; forward: 5'-CTTCCCTGACAGTCACACCC-3', reverse: 5'-TACTACCATTCCCCAGCCGA-3' and as a negative control, targeting GAPDH; forward: 5'-AATGTGTCCGTCGTGGATCTGA-3', reverse: 5'-GATGCCTGCTTCACCACCTTCT-3' were done. The numerical value 3.32 ($log_2$10, representing 10% of input chromatin) was subtracted from the Ct value of the input sample

to generate the adjusted input Ct. Following formula was used to calculate the % input normalized ChIP'ed DNA amount: $100 \times 2^{(\text{Adjusted input Ct} - \text{ChIP Ct})}$.

Western blotting.

Primary hippocampal cultures were treated with leptin (50nM) on DIV6–7. Protein samples were collected from primary hippocampal cultures, HEK293T cells for shRNA efficiency verification, and *ob/ob* or wt mouse hippocampal tissue with RIPA buffer (Santa Cruz Biotechnology) supplemented with phosphatase inhibitor cocktail 2 and 3 (Sigma Aldrich), protease inhibitor cocktail, PMSF (2mM), sodium orthovanadate (1mM, Santa Cruz Biotechnology). The collected samples were centrifuged at 16,000g and debris were discarded. Concentration of samples were measured with BCA assay (Thermo Fisher #23225). Samples were prepared with NuPage LDS Sample Buffer (Life Technologies) and 50mM DTT (Fisher Scientific) and heated at 75°C for 10min. Equal concentration of proteins were then loaded into Bolt 4–12% Bis-Tris gels (Life Technologies). Proteins were transferred to a PVDF membrane (Life Technologies) overnight, blocked with 5% milk, incubated with primary antibodies against KLF4 (Abcam #ab106629, 1:500 and Abcam #72543, 1:250), SOCS3 (Cell Signaling #2932, 1:1000), β-actin (Cell Signaling #4970, 1:1000), c-myc (Sigma #c3956, 1:1000) for 2hrs at room temperature, and then incubated with the appropriate Alexa Fluor-647 secondary IgG $F(ab')_2$ fragment antibody (Cell Signaling, 1:5000) for 1hr at room temperature. Blots were imaged using a Chemidoc MP imaging system (Bio Rad) and analyzed using the ImageJ 1.48 gel analyzer tool. The intensity of target bands in each lane were normalized against the intensity of nuclear protein, β-actin.

Immunostaining.

Transfected neurons were treated and fixed as described above. After fixation, cells were rinsed in PBS and permeabilized with 0.25% Triton X-100 detergent (Bio-Rad Laboratories), followed by 2 rinses in PBS, and blocked with 5% BSA in PBS for 30minutes. Cells were rinsed with PBS again, and incubated with antibodies; (anti-vesicular glutamate transporter (VGLUT)1 (NeuroMab #75–066, 1:250), anti-SOCS3 (Abcam #ab16030, 1:500) or anti-pSTAT3 (Abcam #ab76315, 1:500) diluted in IHC-Tek (#IW-1001) for 2 hours at room temperature with mild shaking. Cells were rinsed with PBS three times and incubated in the appropriate Alexa Fluor secondary IgG antibodies (Thermo Fisher #A31571 and #A11035, 1:1000) for 2 hours at room temperature. Then, they were incubated in Hoechst (Invitrogen #3570, 1:1000) for 10 minutes. They were washed with PBS three times and mounted with Elvanol. Neurons for colocalization analysis were imaged as described in the Spine quantification section and juxtaposition of spines with presynaptic marker were quantified using ImageJ. For analysis of pSTAT3 and SOCS3 fluorescence intensity, in ImageJ, the z-stacks were summed, and background intensity were subtracted. Either whole neuron (Clover) or just nucleus (Hoechst) have been used as a mask and mean fluorescent intensity were reported due to the differences in neuron size.

Quantitative Real-time RT-PCR (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 R^2 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.

Whole-cell recordings.

Patch-clamp experiments were performed on mRFP-β-actin transfected cultured hippocampal neurons with PBS (vehicle control) or 50nM leptin pretreatment. Recordings were made on DIV11 to DIV14. The culture medium was exchanged by an extracellular solution containing 140mM NaCl, 2.5mM KCl, 1mM $MgCl_2$, 3mM $CaCl_2$, 25mM glucose, and 5mM HEPES; pH was adjusted to 7.3 with KOH, and osmolality was adjusted to 306–310 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: 25mM CsCl, 100mM $CsCH_3O_3S$, 10mM phospho-creatine, 0.4mM EGTA, 10mM HEPES, 2mM $MgCl_2$, 0.4mM Mg-ATP, and 0.04mM 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 (1μM; Sigma-Aldrich), and blocking action potential generation with tetrodotoxin (500nM; 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 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 >1 GΩ and neuron input resistance >200 MΩ. The mEPSCs had a 5-min recording time.

Statistical Analysis.

All data were analyzed, and all graphs were generated with SigmaPlot 14.0. First, data sets were tested for normal distribution of the population. When the data sets have normal distribution, to analyze more than two groups, one-way ANOVA followed by Bonferroni

multiple comparison, or to compare two groups, student's t-test were used. When the data sets did not have normal distribution; to analyze more than two groups, Kruskal-Wallis non-parametric ANOVA followed by Dunn's post-hoc analysis was used and p-values are further adjusted by Holm FWER method. All experiments done using primary hippocampal neurons were done on at least 3 independent culture preparations. All data was reported as the mean ± standard error of the mean (SEM). Statistical significance was set to a minimum of $p<0.05$.

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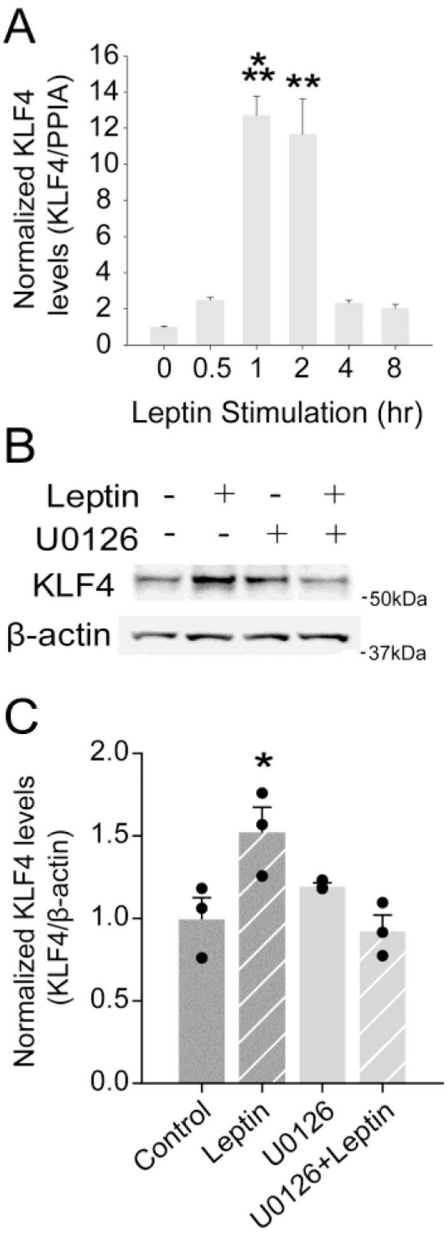

Figure 1:

Leptin increases KLF4 expression in hippocampal neurons. **(A)** DIV6–7 hippocampal neurons were treated with leptin (50nM) for specified durations and processed for qRT-PCR detection of KLF4 mRNA. Average relative fold change in KLF4 mRNA (normalized to PPIA) was graphed. Data were analyzed with Kruskal-Wallis test, followed by Dunn's multiple comparison. **(B, C)** Hippocampal neurons were stimulated with leptin (50nm) and/or U0126 (20μM) for 2 hours. Western blot was done with anti-KLF4 and normalized against β-actin. Data were analyzed with one-way ANOVA, followed by Bonferroni multiple comparison. Data were represented as mean ± SEM. Each dot represents one culture (***$p<0.001$, **$p<0.01$, *$p<0.05$).

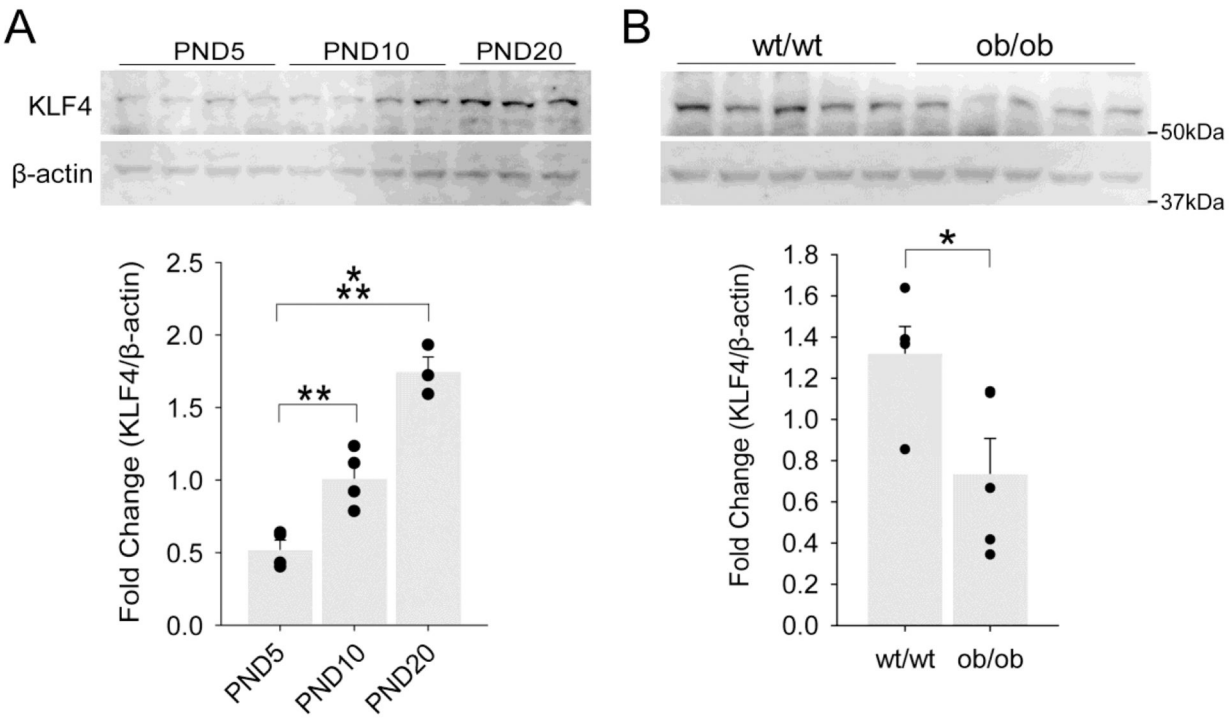

Figure 2:

Leptin regulates KLF4 expression *in vivo*. **(A)** Hippocampal tissues from wild-type animals at the ages of PND5 (n=4), PND10 (n=4), and PND20 (n=3) were harvested. Western blot was done with anti-KLF4 and normalized against β-actin. Data were analyzed with one-way ANOVA followed by Bonferroni multiple comparison. **(B)** Hippocampal tissues from PND10 ob/ob (n=5), and wild-type animals (n=5 littermates of ob/ob animals) were analyzed with western blotting for KLF4 and normalized against β-actin. Data were analyzed with student's t-test. Data were represented as mean ± SEM. Each dot represents one animal (***p<0.001, **p<0.01, *p<0.05).

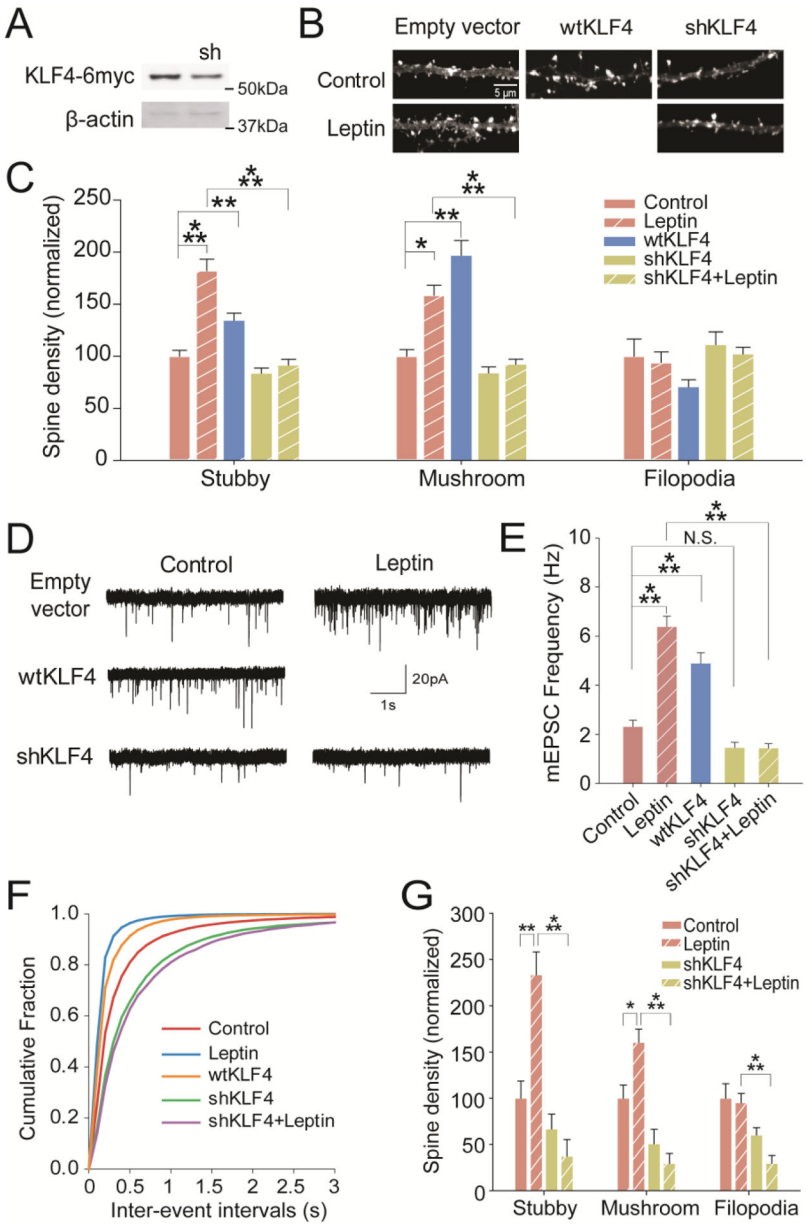

Figure 3:

KLF4 increases functional spine formations in hippocampal neurons. **(A)** HEK293T cells were transfected with KLF4–6myc and shKLF4 construct. Western blotting was done with anti-c-myc and anti-β-actin. **(B, C)** DIV5–6 hippocampal neurons were transfected with Clover-βactin and either wtKLF4 or shKLF4, followed by leptin stimulation on DIV7. Neurons were fixed, and their spine densities were analyzed on DIV12. **(D, E)** Hippocampal neurons were transfected and stimulated as in **B**, and later mEPSCs were recorded. **(F)** Cumulative probability of the frequency of recorded mEPSC events. **(G)** Hippocampal organotypic slice cultures were biolistically transfected with Tomato±KLF4 on DIV2, followed by stimulation with leptin on DIV4. Neurons were fixed, and their spine densities were analyzed on DIV7. All data were analyzed with Kruskal-Wallis test followed

by Dunn's multiple comparison. Data were represented as mean ± SEM (***p<0.001, **p<0.01, *p<0.05).

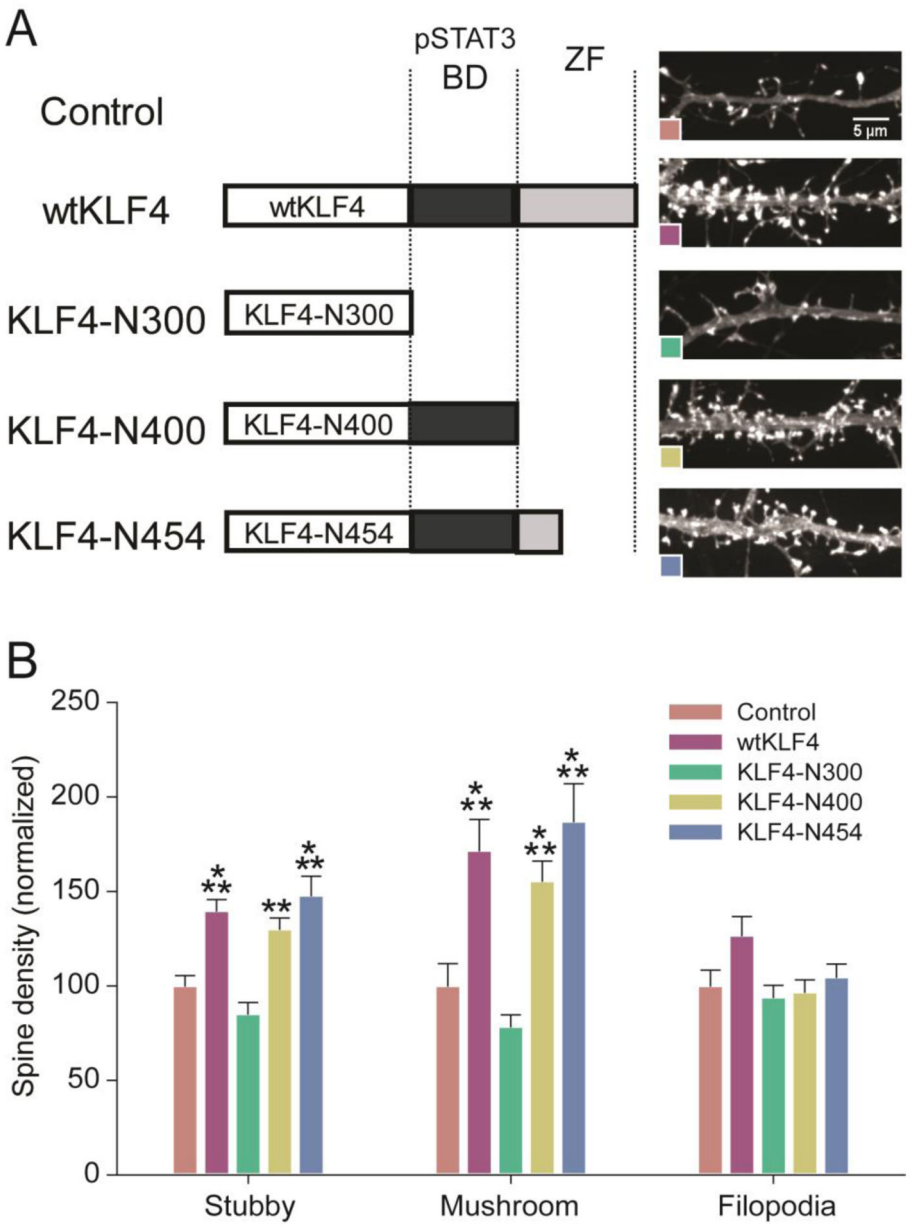

Figure 4:

KLF4 requires the STAT3-binding domain to induce mature spine formation. **(A)** Schematic presentation of different KLF4 deletion constructs. **(B)** DIV5–6 hippocampal neurons were transfected with Clover-βactin along with wtKLF4, KLF4-N400, KLF4-N454, or KLF4-N454. The neurons were fixed, and their spine densities were analyzed on DIV12. All data were analyzed with Kruskal-Wallis test followed by Dunn's multiple comparison. Data were represented as mean ± SEM (***p<0.001, **p<0.01, *p<0.05).

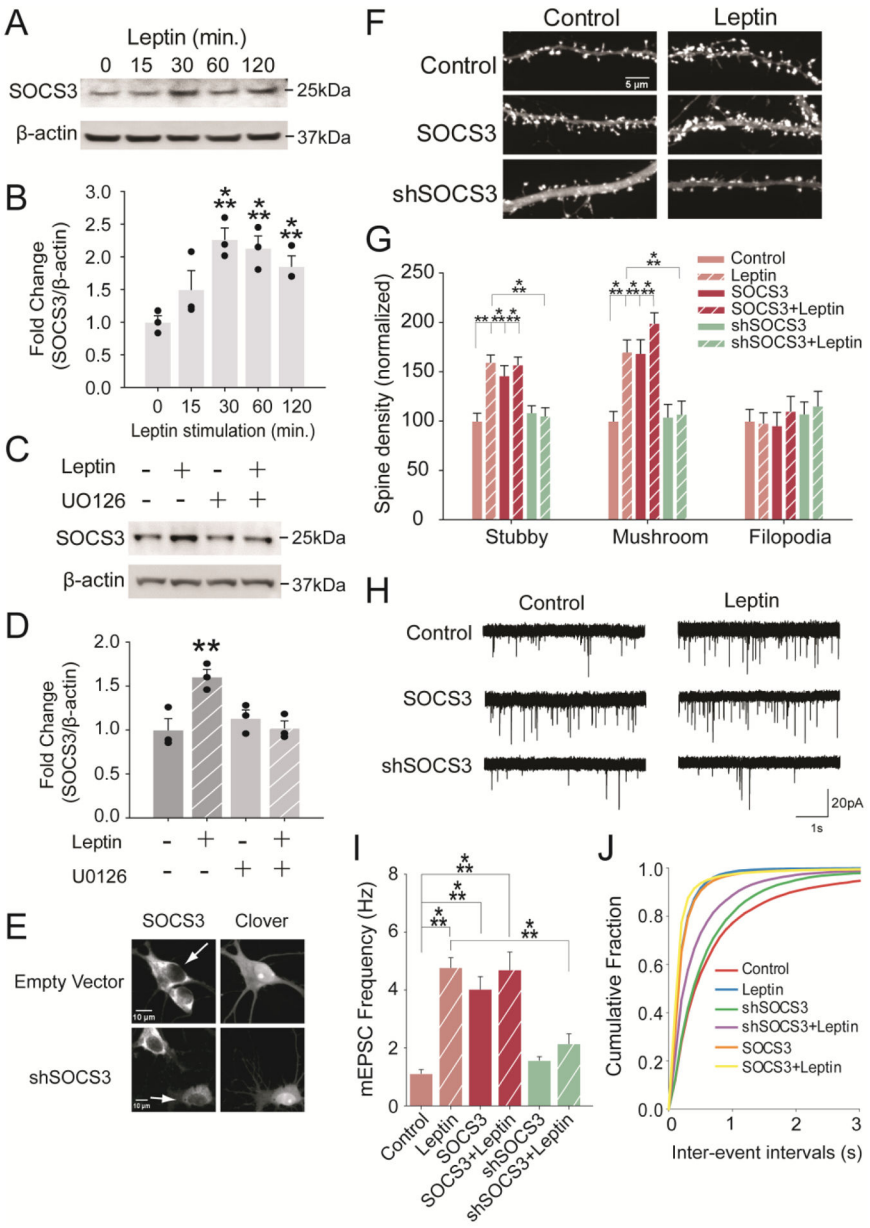

Figure 5:

SOCS3 increases functional spine formations in hippocampal neurons. **(A, B)** Leptin increased SOCS3 expression in DIV6–7 hippocampal neurons as early as 30 minutes of leptin stimulation. **(C, D)** Hippocampal neurons were stimulated with leptin (50nM) and/or U0126 (20μM) for 2 hours. Western blot was done with anti-SOCS3 and normalized against β-actin. All western blot data were analyzed with one-way ANOVA followed by Bonferroni multiple comparison. **(E)** Hippocampal neurons were transfected with pCAGGS-Clover ± shSOCS3 construct. Neurons were stained with anti-SOCS3. Transfected neurons were indicated with arrows. Note that in the upper left panel, there was no difference in SOCS3 levels between non-transfected and only pCAGGS-Clover (empty vector) transfected neurons. **(F, G)** DIV5–6 hippocampal neurons were transfected with Clover-βactin and

either SOCS3 or shSOCS3, followed by leptin stimulation on DIV7. Neurons were fixed and analyzed on DIV12. **(H, I)** Hippocampal neurons were transfected and stimulated as in **G**, and mEPSCs were recorded. **(J)** Cumulative probability of the frequency of recorded mEPSC events. Both spine and electrophysiology data were analyzed with Kruskal-Wallis test followed by Dunn's multiple comparison. Data were represented as mean ± SEM. Each dot represents one culture (***$p<0.001$, **$p<0.01$, *$p<0.05$).

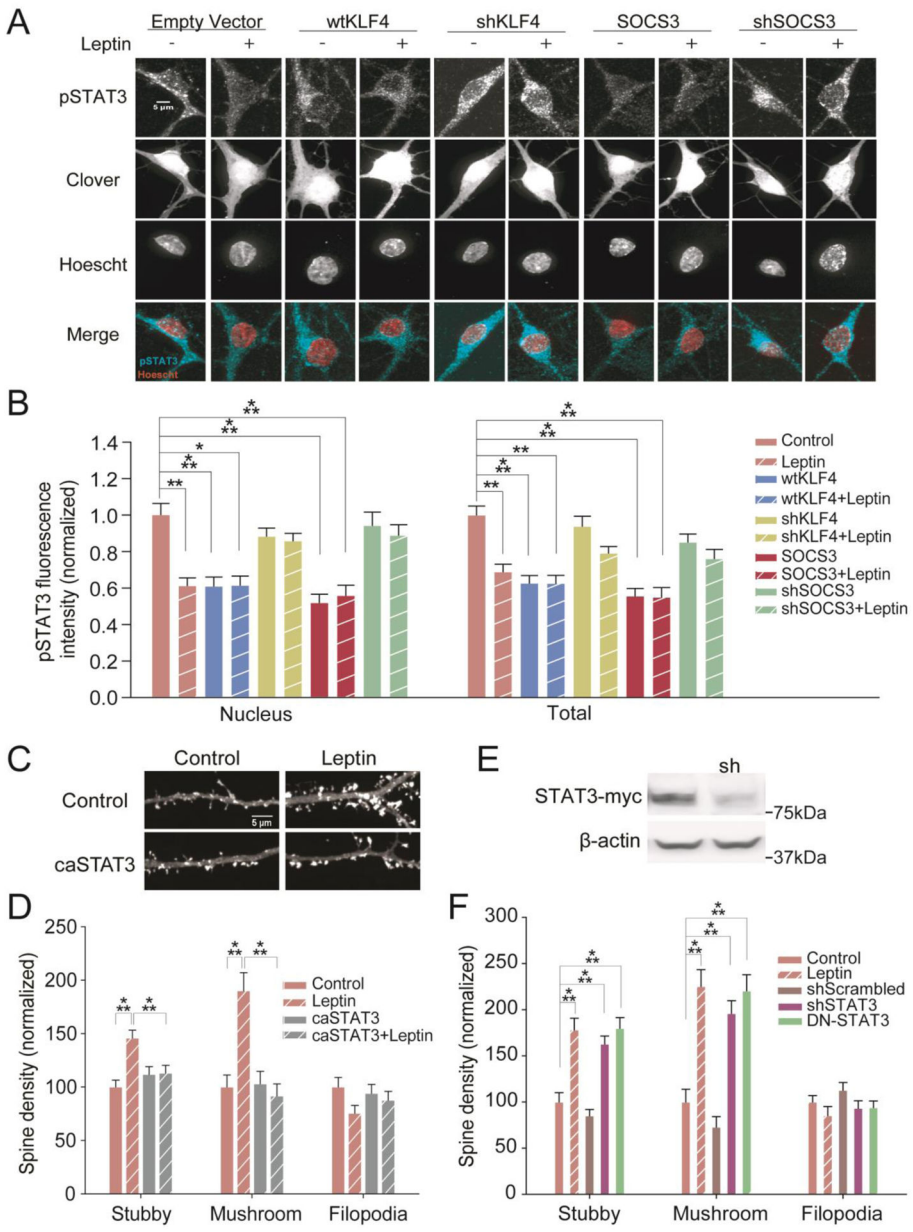

Figure 6:

Both KLF4 and SOCS3 decrease STAT3 activity and inhibition of STAT3 increases spinogenesis. **(A, B)** DIV5–6 hippocampal neurons were transfected with pCAGGS-Clover and either wtKLF4, shKLF4, SOCS3, or shSOCS3. After 2 hours of leptin treatment on DIV7, neurons were fixed and stained for pSTAT3. **(C,D)** Hippocampal neurons were transfected on DIV5–6 with Clover-βactin ± caSTAT3, followed by leptin stimulation on DIV7. Neurons were fixed and analyzed on DIV12. These data were analyzed with Kruskal-Wallis test followed by Dunn's multiple comparison. **(E)** HEK293T cells were transfected with STAT3-myc and ±shSTAT3 construct. Western blotting was done with anti-c-myc and anti-β-actin antibodies to show the efficiency of knockdown. **(F)** DIV5–6 hippocampal neurons were transfected with Clover-βactin and either shSTAT3, scrambled shRNA, or

dnSTAT3, followed by leptin stimulation on DIV7. Neurons were fixed and analyzed on DIV12. All data were analyzed with Kruskal-Wallis test followed by Dunn's multiple comparison. Data were represented as mean ± SEM (***p<0.001, **p<0.01, *p<0.05).

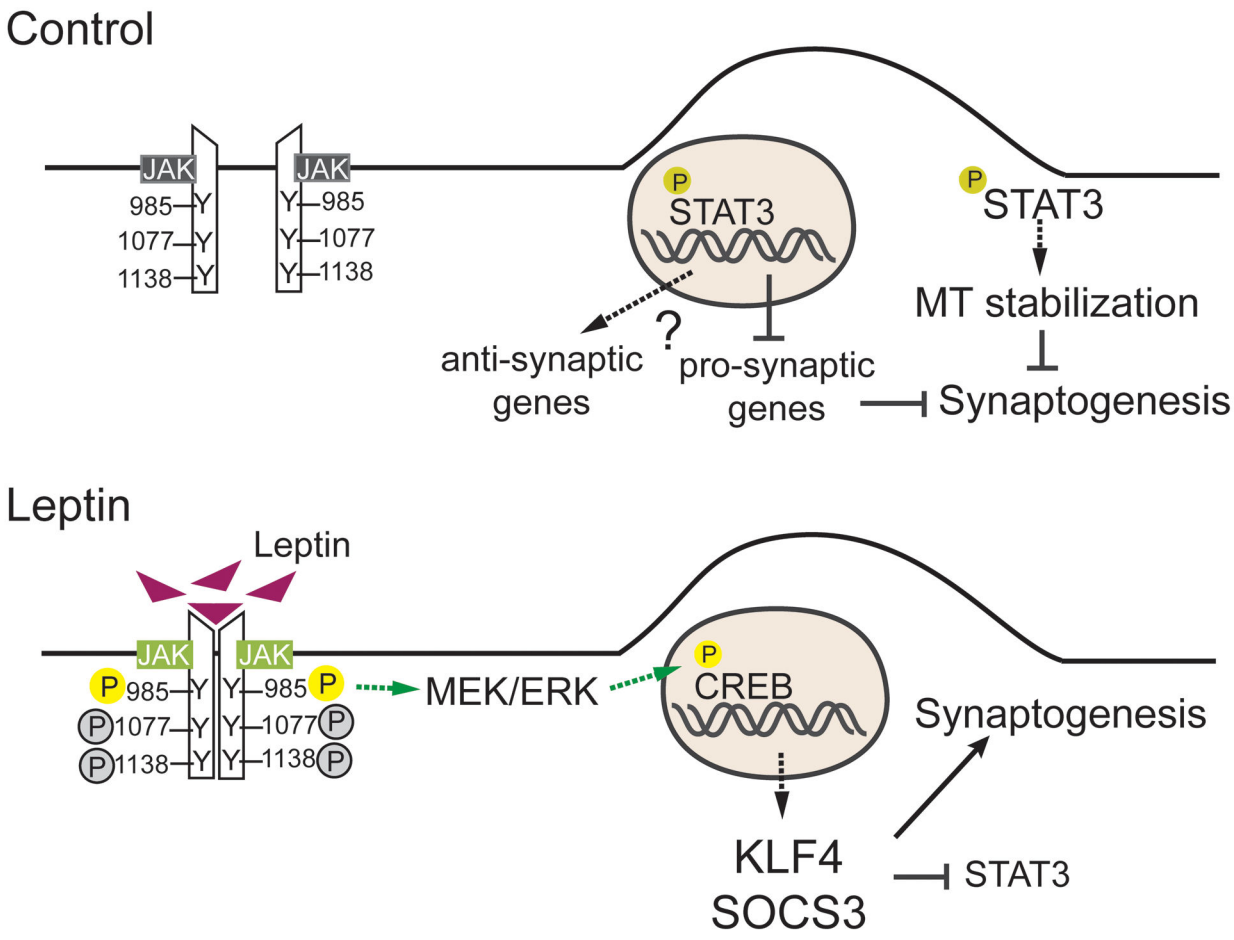

Figure 7:

Model of current hypothesis. In control conditions, STAT3 is present at basal levels, and this acts as a brake on glutamatergic synaptogenesis. When the leptin receptor is activated in the presence of leptin, STAT3 activity is inhibited by the upregulation of KLF4 and SOCS3; this removes the brake and leads the formation of functional glutamatergic synapses in hippocampal neurons. MT: microtubules.