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THE IDENTIFICATION OF THE DIRECT AND INDIRECT PATHWAYS THROUGH WHICH LEPTIN FACILITATES SYNAPTIC PLASTICITY IN THE HIPPOCAMPUS

by

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ABSTRACT

Leptin, a peptide synthesized by adipocytes in the periphery, has been shown to play significant roles in feeding and energy expenditure mediated by the hypothalamus. Growing evidence supports the role of leptin in influencing synaptic plasticity in the hippocampus in that leptin dose-dependently enhances LTP, alters morphology and neurogenesis, facilitates spatial learning and memory, as well as memory retention. Models of leptin deficiency and resistance have further supported the importance of leptin in synaptic plasticity by exhibiting deficits in electrophysiological, morphological and behavioral tests that are improved after leptin restoration. The effects of leptin when applied directly into the hippocampus have clear effects on synaptic plasticity; however, indirect pathways may also be activated by leptin that result in synaptic plasticity changes in the hippocampus. Due to the nature of the release of leptin from the peripheral adipocytes, the interconnectivity of the brain, and the localization of leptin receptors throughout many brain regions, support the hypothesis that leptin could mediate synaptic plasticity changes in the hippocampus through multi-synaptic circuitry in addition to its direct effects. A variety of brain regions that express leptin receptors project both directly and indirectly to the hippocampus, including many nuclei of the hypothalamus, raphe nucleus, locus coeruleus and ventral tegmental area and therefore pose as potential starting points for brain-wide networks that leptin could influence for the regulation of synaptic plasticity in the hippocampus.

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CHAPTER 1

DIRECT EFFECTS OF LEPTIN IN THE HIPPOCAMPUS

1.1 SYNAPTIC PLASTICITY: AN INTRODUCTION

Synaptic plasticity may be defined as a modification in synaptic strength after a repeated stimulus. Changes in synaptic plasticity form the basis for how the brain is able to adapt itself in response to stimuli and is part of the proposed mechanism for learning and memory. The phenomenon of synaptic plasticity is important because it results in a change in efficacy in how neurons respond to a stimulus and therefore the response to the stimulus changes. Synaptic plasticity is thought to underlie certain types of learning and memory and this is demonstrated in the hippocampus, a well-studied structure shown to be integral in particular spatial learning and memory.

Synaptic plasticity has many aspects that can be observed and regulated, both positively and negatively. Factors such as stress (Gould et al, 1997; Schoenfeld and Gould, 2012; Yap et al, 2006) and aging (Kuhn et al, 1996; Seki and Arai, 1995) can detrimentally affect learning and memory. Conversely, estrogen (Gould, 1999), the neurotransmitter serotonin (Brezun and Daszuta, 1999), an enriched environment (Nilsson et al, 1999) and exercise (Praag et al, 1999) can positively modulate synaptic plasticity. The 167 amino acid peptide leptin has also been shown to influence synaptic plasticity (Maffei et al, 1995).

Synaptic plasticity in the hippocampus can be directly altered, for example by applying a stimulus directly to neurons to see an enhancement of synaptic plasticity.

However, synaptic plasticity also be indirectly affected, through receptors in different brain areas projecting to others in such a way that initiation at one area eventually leads to the hippocampus to alter synaptic activity. This multi-synaptic circuitry must operate through a series of neurons connected by synapses that are inhibited or excited by neurotransmitters. Leptin receptors are expressed in regions that have projections to the hippocampus and in this manner leptin may be uniquely positioned to directly and indirectly affect synaptic plasticity in the hippocampus.

1.2 LEPTIN, LEPTIN RECEPTORS & SIGNALING

Leptin, the protein product of the obesity (*ob*) gene, is predominantly synthesized by adipocytes, proportional to the amount of adipocytes in the body (Frederich et al, 1995). Although leptin is primarily synthesized in the periphery, there is some evidence that it can be synthesized in the brain as there is evidence of the presence of leptin mRNA and protein in some brain regions, including the cerebral cortex and hypothalamus (Elmquist et al, 1998). Leptin mRNA transcription in the hypothalamus has been shown to be blunted when fasting occurs and leptin levels have also been shown to fall during starvation (Morash et al, 1999; Frederich et al, 1995).

Once leptin is released into circulation, for example after eating, leptin can act in the periphery to play roles in immunity, (Lord et al, 1998; Oomura et al, 2006), inhibit insulin secretion, (Bjorbaek and Kahn, 2004), control sex hormone release (Yu et al, 1997; Ahima et al, 1996) and lipolysis in adipocytes (Siegrist-Kaiser et al, 1997; Wang et al, 1999). Leptin receptors in the body have been found in skeletal muscle, heart, lungs, liver, white adipose tissue, adrenal glands and the kidneys (Tartaglia et al, 1995; Lee et

al, 1996; Fei et al, 1997; Ghilardi and Skoda, 1997; Hoggard et al 1997; Bjorbaek and Kahn, 2004). Peripheral leptin receptors are expressed as the short form of the receptor, ObRa, and while the exact functions of peripheral ObRa are still not known, there is evidence of signaling in pancreatic β cells, pituitary cells, hepatocytes, muscle cells and adipocytes (Hileman et al, 2002; Bjorbaek and Kahn, 2004). Leptin has been shown to activate PI3K pathways for signaling in insulinoma cells (Harvey et al, 2000), hepatocytes (Zhao et al, 2000), and muscle cells (Berti et al, 1997).

Leptin can also cross the blood-brain barrier (BBB) through a saturable transport system to then act in the central nervous system (CNS) (Banks et al, 1996; Hileman et al, 2002; Banks et al, 2006). The role of leptin in the CNS has been described as being regulatory, where leptin acts as a retrograde modulator (Harvey et al, 2007; Ludwig and Pittman, 2003). In the CNS, leptin activates the long isoform of the leptin receptor, the ObRb, a class 1 cytokine receptor encoded by the diabetes (*db*) gene (Tartaglia et al, 1995). There are six leptin receptor isoforms; ObRa, ObRb, ObRc, ObRd, ObRe and ObRf but only the long isoform, ObRb, is capable of complete JAK/STAT signal transduction (Baumann et al, 1996; Ghilardi et al, 1996; Bahrenberg et al, 2002; Tartaglia et al, 1995).

The ObRb is often paired with the JAK/STAT specific subtypes, JAK2/STAT3, JAK2/STAT5, and JAK2/STAT6 (Ghilardi et al, 1996) although other pathways can be used including the MAPK, and PI3K pathways (Bjorbaek et al, 2004; Niswender et al, 2001; Zhao et al, 2002; Rahmouni et al, 2003; Cota et al, 2006, 2008).

In JAK/STAT signaling, leptin binds to its receptor and initiates a conformational change to phosphorylate tyrosine residues on JAK2. The now phosphorylated JAK2

stimulates phosphorylation of tyrosine residues on the ObRb receptor, which then recruits Src-homology2 and Src-homology 3 that contain within them signal transducer and activator of transcription 5 (STAT5) and STAT3. STAT5 and STAT3 are then in turn phosphorylated by JAK2 and STAT5 and STAT3 can now separate off of the ObRb and dimerize, moving into the nuclear envelope for gene transcription regulation (Marwarha and Ghribi, 2012). Phosphorylated tyrosine residues can recruit STAT family transcription factors, phosphoinositide 3-kinase and proteins in the MAPK/Ras Raf signaling pathways (Tartaglia et al, 1995; Harvey, 2003).

The MAPK pathway also involves phosphorylation of tyrosine residues and downstream processes similar to JAK/STAT signaling and can similarly regulate gene expression in the CNS (Marwarha and Ghribi, 2012). The PI3K pathway is also activated by leptin signaling with the ObRb and utilizes the phosphorylation of Akt at serine residues in a similar process to that of JAK/STAT signaling (Vecchoine et al, 2002). Studies have also indicated that leptin can activate the AMPK-SIRT1 pathway (Yamagishi et al, 2001; Minokoshi et al, 2002; Suzuki et al, 2007).

JAK/STAT pathway has been shown to be critical in the actions of leptin in body weight regulation (Bates et al, 2003). PI3K signaling is proposed to be involved in leptin-stimulated reductions in food intake as well as activation of sympathetic nervous system activity (Zhao et al, 2002b; Rahmouni et al, 2003).

Leptin receptor mRNA has been found in many different brain regions including the pituitary, arcuate nucleus, dorsomedial hypothalamus (DMH), ventromedial nucleus (VMH), ventral premammillary nucleus, lateral hypothalamus, thalamic nuclei, cerebellum and choroid plexus and the hippocampus (Elmquist et al, 1998; Morash et al,

1999). Using *in situ* hybridization, it was found that specific areas of the hypothalamus show high levels of leptin receptor expression, including the arcuate nucleus, DMH and VMH (Schwartz et al, 1996). Additionally, specific areas of the hippocampus also have high levels of immunoreactivity of the leptin receptor, including areas CA1, CA2, CA3 and the dentate gyrus in particular on neurons and glial cells (Ur et al, 2002; Mercer et al, 1996; Hakansson et al, 1998; Huang et al, 1996; Shanley et al, 2002). Hippocampal cell culture studies have allowed for the localization of leptin receptor expression and have revealed that leptin receptors are expressed on the dendrites and axons of neurons, in close proximity to NMDA receptors (Shanley et al, 2002b; O'Malley et al, 2007).

In contrast to the ObRb's role in signaling, the ObRa short-form leptin receptor may be involved in receptor-mediated transport of leptin across the BBB (Hileman et al, 2002). The ObRa mRNA is found in high levels in the choroid plexus and brain microvessels of the BBB (Tartaglia et al, 1995; Bjorbaek et al, 1998). In support of this hypothesis, knock out mice for short form leptin receptors resulted in decreased leptin transport across the BBB (Bjorbaek et al, 1998; Hileman et al, 2002). These results suggest that the ObRb is the main signaling receptor isoform in the brain while the ObRa is used for transport across the BBB.

1.2.1 LEPTIN IN THE HYPOTHALAMUS: FUNCTIONS IN FEEDING & ENERGY EXPENDITURE

There are many well-studied functions that leptin regulates through activation of leptin receptors expressed in the hypothalamus, including regulation of metabolism, energy homeostasis, body composition and feeding behaviors (Zhang et al, 1994; Ahima et al, 1996; Elmquist, 1999; Elias et al, 1999). Triglycerides are deposited into adipocytes

in amounts associated with how much food is being consumed and the amount of stored triglycerides determine how much leptin mRNA and plasma leptin protein is produced (Kulcsar et al, 2005). As such, leptin serves as an adiposity indicator, secreted in proportion to the amount of fat stored and higher levels of leptin decrease food intake and increase energy expenditure (Schwartz et al, 2000). Direct injection of leptin in animals lacking leptin or proper leptin receptor functioning into the hypothalamus resulted in a significant decrease in feeding as well as fat and weight loss (Zhang et al, 1994; Morton et al, 2006).

Leptin suppresses food-seeking behaviors by inhibiting neuropeptide Y (NPY)/AgRP and orexin neurons and activating POMC/CART neurons in the arcuate nucleus (Huang et al, 1996; Cowley et al, 2001). These populations of neurons project to the lateral hypothalamus for well-studied control of food intake (Elias et al, 1999; Mercer et al, 1996a; Cheung et al, 1997). These roles of leptin in the regulation of feeding and energy metabolism mediated through the lateral and arcuate nuclei of hypothalamus lend understanding to the functional activity of leptin outside of the hypothalamus in brain regions such as the hippocampus.

1.3 THE HIPPOCAMPUS

As noted above, the ObRb mRNA and protein are expressed in many brain regions including the hippocampus (Hakansson et al, 1998; Elmquist et al, 1998; Haung et al, 1996). The hippocampus, part of the limbic system involved in learning and memory, is comprised of layers of pyramidal cells which are then divided into different areas, Cornu Ammonis regions 1-4 (CA1-4). The structure of the hippocampus is such

that information flows in one direction and follows a series of pathways. Information enters the hippocampus from the entorhinal cortex through the perforant path to the dentate gyrus. Axons project from the dentate gyrus, projections called the Mossy Fiber Pathway, to area CA3 of the hippocampus, also pyramidal neurons. Projections from area CA3, the Schaffer Collaterals/Commissural Pathway, project to area CA1 which then projects to the subiculum and entorhinal cortex and exit the hippocampus. Leptin receptors are found in areas CA1, CA3 and the dentate gyrus, areas where synaptic plasticity has been shown to occur (Ur et al, 2002).

CHAPTER 2

LEPTIN & SYNAPTIC PLASTICITY IN THE HIPPOCAMPUS

2.1 LEPTIN & LTP

Long-term potentiation (LTP) and long-term depression (LTD) are activity-dependent forms of hippocampal synaptic plasticity and are considered as being cellular correlates for learning and memory, or the most fundamental units of measuring learning and memory. LTP is a greater response and LTD is a lesser response of a neuron that occurs after high-frequency stimulation, as assessed using electrophysiological techniques (Bliss and Lomo, 1973; Bliss and Gardner-Medwin, 1973). Electrophysiology is a technique used to measure electrical activity in neurons or cells. A glass micropipette filled is inserted into a neuron or cell and another electrode is inserted into the surrounding fluid around the cell for reference. Voltage can be clamped to hold the membrane potential so that current can be measured. Current can be clamped in a similar fashion so that current is applied and membrane potential can be measured. Using different electrophysiology clamping and recording techniques, it is possible to determine when channels are opened by a stimulus or when neurons are activated.

LTP is important in learning and memory, as evidenced by when LTP induction is blocked, impairments in memory are seen (Grant and Silva, 1994). Mutations in a gene encoding a particular tyrosine kinase involved in LTP resulted in impairments in LTP in area CA1 as well as spatial learning deficits, demonstrating the importance of LTP in spatial learning (Grant et al, 1992; Bliss and Collingridge, 1993).

LTP occurs at all excitatory synapses in the hippocampus and other areas of the brain (Doyere et al, 1992). LTP has been shown to last for hours in hippocampal slices and days in animals that have had LTP induced (Bliss and Collingridge, 1993). The enhanced response seen by a neuron after high-frequency stimulation occurs is proposed as being a combination of several events, which may include increased neurotransmitter release by the presynaptic neuron, increased number of receptors localized to the activation of the synapse and less uptake of neurotransmitter by nearby glial cells (Bliss and Collingridge, 1993).

LTP can be *N*-methyl-D-aspartate (NMDA) receptor-dependent in the Perforant pathway and Schaffer collaterals in the hippocampus and also NMDA receptor-independent in the Mossy Fiber pathways (Bliss and Collingridge, 1993). The NMDA receptor is a type of glutamate receptor and is involved in synaptic plasticity in the hippocampus (Lisman et al, 2012). NMDA antagonists block LTP induction, demonstrating the necessity of NMDA receptors in initializing LTP (Bliss and Collingridge, 1993).

NMDA receptor activation is critical for LTP to occur, the more the NMDA receptors involved in propagating signal, the more likely that LTP will initiate in the perforant pathway and Schaffer collaterals, those pathways whose LTP is NMDA receptor-dependent (Malenka, 1994; Collingridge et al, 1983). NMDA-dependent LTP is made possible due to several unique properties of the receptor itself. NMDA receptors contain a magnesium plug that blocks the channel until the membrane potential is sufficiently depolarized and thus the NMDA receptor is referred to as being voltage-dependent (Ascher and Nowak, 1988). In addition, simultaneous glutamate binding to

NMDA receptor must occur and so the NMDA receptor is a 'coincidence detector' due to the several conditions that must be met at once for the channel to open (Ascher and Nowak, 1988). Once the channel is open, calcium flows through and increases intracellular calcium, beginning a cascade of signaling events that lead to LTP. As intracellular calcium levels increase, calcium binds to calmodulin and this complex activates calcium/calmodulin-dependent protein kinase II (CaMKII), which has been identified as an important component of NMDA-mediated LTP (Lisman et al, 2012). CaMKII is important in the induction of LTP and knockdown of an isoform of CaMKII has been shown to impair hippocampal slice LTP (Silva et al, 1992a; Malinow et al, 1989). Leptin can influence many aspects of LTP and LTD mediated through ObRb receptors in the CA1, CA3 and the dentate gyrus regions of the hippocampus (Mercer et al, 1996; Oomura et al, 2006; Shanley et al, 2001; Shanley et al, 2002; Xu et al, 2008).

Leptin application shifts short-term potentiation (STP) to LTP (Shanley et al, 2001). STP is similar to LTP in that it is an increased response to tetanus stimulation, however it persists for only seconds to minutes. Hippocampal slices were used to show STP conversion after stimulation of the Schaffer collateral commissural pathway (Shanley et al, 2001; Harvey et al, 2007). STP was evoked in hippocampal slices that lasted about 30 minutes (Shanley et al, 2001). If the slice was incubated with leptin immediately preceding the primed burst stimulation to evoke STP, then STP was converted into LTP that lasted for an hour (Shanley et al, 2001).

Leptin enhances and suppresses NMDA receptor-dependent LTP in a dose-dependent manner (Oomura et al, 2006; Wayner et al, 2004). Electrophysiology studies at the Schaeffer collaterals in the hippocampus showed that leptin application resulted in

enhancement of LTP by 163% compared to controls (Oomura et al, 2006). Leptin application dose-dependently enhanced LTP with the lowest and highest doses resulting in reducing LTP, resulting in an inverted U dose response (Oomura et al, 2006). Leptin also enhances LTP in other areas of the hippocampus, including the dentate gyrus (Wayner et al, 2004). Hippocampal slice studies show similar results, LTP was induced by bath administration of leptin (Li et al, 2002; Shanley et al, 2001; Wayner et al, 2004). Interestingly, age can affect the ability of leptin to increase excitatory transmission. In this regard, it was shown that in hippocampus slices from juvenile rodents that leptin decreases excitatory transmission, which is contrary to what occurs in adults (Moult et al, 2010; Moult and Harvey, 2011). This has been proposed to being related to the process of brain development (Irving and Harvey, 2014).

In addition to changes in LTP, changes in excitatory postsynaptic potentials (EPSCs) were found with leptin application. Leptin receptor signaling resulted in increases in NMDA receptor excitatory postsynaptic currents (EPSCs) in area CA1 but decreases AMPA receptor EPSCs (Shanley et al, 2001). In further support, electrophysiological studies for field potentials in CA1 pyramidal neurons revealed significant increases in mEPSC amplitude through NMDA receptors dependent on the dose of leptin (Oomura et al, 2006). It has been shown that leptin receptors are required for this effect by using *Xenopus* oocytes with variable NMDA receptor composition and the ObRb.

Leptin enhanced NMDA EPSCs with the NR1/NR2A subunits of the NMDA receptor with the ObRb present (Shanley et al, 2001). When the ObRb was eliminated, leptin did not enhance currents with the NMDA subunits NR1/NR2A when NMDA was

applied (Shanley et al, 2001). It has been proposed that leptin may traffic NMDA receptors to the membrane to affect the increased current through NMDA receptors, just as insulin has been shown to do (Shanley et al, 2001; Harvey et al, 2007; Skeberdis et al, 2001). These studies collectively show that leptin and the leptin receptor directly effect LTP and EPSCs through several mechanisms in the hippocampus.

Leptin enhances calcium influx through NMDA receptors and also increases the activity of CaMKII (Shanley et al, 2001; Oomura et al, 2006). Calcium influx into postsynaptic neurons is vital in the induction step of LTP and leptin is able to increase calcium flux into hippocampal CA1 neurons via NMDA receptors (Bliss and Collingridge, 1983; Oomura et al, 2006). Calcium concentration was measured in single neurons from area CA1 of the hippocampus in response to leptin. Leptin application dose-dependently resulted in increases in calcium in the same inverted U dose response as seen with LTP (Oomura et al, 2006).

It was also determined that leptin increases calcium flow through NMDA receptors as part of the induction phase of LTP (Shanley et al, 2001). Hippocampal cell cultures showed that leptin quickly enhanced inward flow of calcium through NMDA receptors in postsynaptic neurons after NMDA was applied (Shanley et al, 2001). To further support the ability of leptin to increase calcium mediated by NMDA receptors, it was shown that without the NMDA receptor agonist NMDA, leptin did not enhance calcium levels (Shanley et al, 2001). Additionally, leptin had no effect on calcium increase facilitated by AMPA receptors (Shanley et al, 2001). To determine which signaling pathways were involved, PI3K signaling was blocked with LY 294002 and wortmannin which resulted in a significant reduction in the ability of leptin to enhance

calcium influx into neurons. Similar experiments blocking MAPK signaling with PD 98059 and U0126 resulted in a reduction of the ability of leptin to enhance NMDA currents as well as calcium influx (Shanley et al, 2001). This supports the idea that leptin increases calcium through MAPK and PI3K-dependent mechanisms.

CaMKII is an enzyme involved in signal transduction in LTP and has been shown to be involved in LTP maintenance as well as play critical roles in learning and memory (Fukunaga et al, 1993; Silva et al, 1992; Oomura et al, 2006). CaMKII activity was assessed using an assay and results showed that leptin at the same dose that increased calcium concentration increases CaMKII activity, whereas this effect on CaMKII was not seen at other doses of leptin (Oomura et al, 2006). These data collectively show that leptin increases calcium influx through NMDA receptors in the postsynaptic neuron and increases CaMKII activity dose-dependently (Oomura et al, 2006).

Leptin can also reverse LTP, a process called depotentiation, at area CA1. Depotentiation is important in preventing potentiated synapses from becoming saturated and to aid neurons in storing information (Harvey, 2013; Moult et al, 2009). The ability of leptin to depotentiate synapses is dependent on the dose administered and time course (Moult et al, 2009). Rapid depotentiation was seen with the highest doses of leptin compared to lowest doses of leptin. The time after LTP induction is also important, 30 minutes after LTP was induced, leptin was given and LTP was completely reversed. However, leptin administrated after 50 minutes did not change LTP (Moult et al, 2009). This reversal is NMDA-receptor dependent and involves rectification of currents through AMPA receptors which has been proposed as being a result of an alteration of subunits of AMPA receptors (Moult et al, 2009).

Leptin can also induce NMDA receptor-dependent LTD when excitability is enhanced (Durakoglugil et al, 2005). LTD is the weakening of synapses after repeated stimulus and has been identified as an important aspect of some forms of learning and memory processing (Bear and Abraham, 1996). Part of the mechanism of the lesser response with LTD has been proposed to involve withdrawing AMPA receptors from the membrane and the reduction of dendritic spines (Nagerl et al, 2004). GABA A receptors normally inhibit the activation of NMDA receptors due to its ability to increase the intensity of the magnesium blocking of the NMDA receptor. In hippocampal slices with magnesium removed or with picrotoxin, a GABA A receptor antagonist, leptin application resulted in LTD. In the same study, it was shown that NMDA receptors are required for the LTD to occur (Durakoglugil et al, 2005). Additionally, wortmannin and LY294002, blockers of PI3K signaling, further increased the depressive response induced by leptin. It can then be inferred that PI3K signaling is involved in inhibiting leptin-induced LTD (Durakoglugil et al, 2005).

Leptin binding to its receptors can activate three different signaling pathways linked to the ObRb: JAK/STAT, PI3K and MAPK (Ghilardi et al, 1996; Banks et al, 2000; Bjorbaek et al, 2000; Niswender et al, 2001; Zhao et al, 2002; Rahmouni et al, 2003; Cota et al, 2006, 2008). However, in regard to synaptic plasticity changes, the JAK/STAT pathway is the unlikely candidate for the signaling pathway because JAK/STAT initiates gene transcription and synaptic changes occur rapidly. Instead, there is evidence that leptin utilizes the PI3K and MAPK pathways for signaling that alters NMDA receptor function due to the ability of these pathways to have fast-acting

responses (Shanley et al, 2001). In the hippocampus, neurons express PI3K (Folli et al, 1994) and MAPK signaling (Fiore et al, 1993).

PI 3-kinase inhibitors LY 294002 and wortmannin with leptin decreased the ability of leptin to facilitate NMDA-receptor dependent calcium increases (Shanley et al, 2001). This demonstrates that the ability of leptin to enhance the increase of calcium through NMDA receptors is dependent on PI3K signaling. MAPK signaling pathways have been shown to play a role in hippocampal synaptic plasticity (Impey et al, 1999; Rosenblum et al, 2000). One study inhibited MEK activity using PD 98059 and U0126 to study if MAPK pathways were being utilized by leptin for some of the rapid NMDA EPSC changes. Blocking MEK with either PD 98059 or U0126 decreased the enhancement of NMDA receptor currents by leptin (Shanley et al, 2001). From these studies that inhibited either PI3K or MEK in the MAPK pathways, it can be concluded that leptin uses these pathways for at least some of the rapid synaptic plasticity changes seen in the hippocampus.

To further support this idea, experiments were completed in the arcuate nucleus to demonstrate the rapid effects of PI3K signaling by leptin to depolarize POMC neurons. PI3K is activated by phosphorylated IRS proteins so that the PI3K enzyme can in turn phosphorylate phosphatidylinositol to have downstream effects (Shyng and Nichols, 1998). Loose-patch recordings of POMC neurons showed that leptin application increases firing rate, as seen in other experiments (Hill et al, 2008; Williams and Smith, 2006; Williams et al, 2007; van den Top et al, 2004; Munzberg et al, 2007). Transgenic mice with impaired PI3K signaling in POMC neurons provided additional evidence that PI3K is required by leptin for rapid signaling by showing that leptin application failed to

increase firing rates of POMC neurons (Hill et al, 2008). Additionally, wortmannin blocked the effects of leptin on firing rate of POMC neurons whereas a MAPK inhibitor did not (Hill et al, 2008). Therefore, leptin signals with the PI3K pathway in the hypothalamus to rapidly depolarize POMC neurons. Although this study was conducted in the hypothalamus, the data show that leptin can signal quickly with PI3K which is similar to what has been found in the hippocampus (Shanley et al, 2001).

Enhancing NMDA responses, as leptin has been shown to do, involves PI3K, MAPK signaling and Src tyrosine kinases (Shanley et al, 2001). This is evidenced by experiments showing that the inhibition of tyrosine kinases blocks the induction of LTP (Salter, 1998; O'Dell et al, 1991). Phosphorylation and Src tyrosine kinases are involved in the improvement of NMDA function (Yu et al, 1997; Zheng et al, 1998; Salter et al, 1998; MacDonald et al, 1989; O'Dell et al, 1991). Using lavendustin A to block tyrosine kinases in the hippocampus reduced the ability of leptin to enhance calcium influx mediated by NMDA receptors (Shanley et al, 2001). If Src tyrosine kinase inhibitors were applied in hippocampal slices, leptin application no longer resulted in increased EPSCs. These data imply that Src tyrosine kinases are involved in the ability of leptin to enhance EPSCs and calcium influx (Shanley et al, 2001). It is clear, then, that tyrosine kinases play an important role in NMDA receptor function and leptin-mediated enhancement of NMDA receptor functions.

In addition to leptin influencing glutamatergic receptors, such as NMDA and AMPA receptors, leptin has been shown to increase GABAergic synaptic transmission onto CA3 neurons in rats (Shanley et al, 2001; Guimond et al, 2014). Whole-cell patch clamping studies on CA3 pyramidal neurons showed that miniature GABA A receptor-

mediated postsynaptic currents were increased after leptin was added to the perfusion solution (Guimond et al, 2014). Further studies concluded that leptin achieves this through calcium influx in the postsynaptic neuron involving the PI3K and MAPK signaling pathways (Guimond et al, 2014). In addition, leptin can also affect GABAergic transmission onto CA1 pyramidal neurons. Hippocampal slices were used to determine the effect of bath application of leptin on inhibitory postsynaptic current (IPSC) amplitude. This effect was also dose-dependent with the higher doses of leptin resulting in a higher evoked IPSC amplitude whereas lower doses had no effect. The heightened IPSC amplitude disappeared after leptin was washed out and so was not a long-term effect (Solovyova et al, 2009). Inhibition of PI3K signaling blocked this leptin-induced increase in IPSC amplitude. These studies add to the existing body of literature that leptin is neuromodulatory and can modulate many different types of neurotransmission, including glutamatergic and GABAergic transmission.

Leptin can also alter glutamate receptor trafficking to the membrane which is important in LTP. For example, leptin treatment of hippocampal slices increased the expression of the GluR1 and GluR2 AMPA receptor subunit in the cell surface via exocytosis (Moult et al, 2010). Additionally, leptin increases the amount of AMPA receptors that are permeable to calcium which lead to an increase in phosphatidylinositol trisphosphate (PIP3). This is proposed as being a part of the process of GluR1 trafficking to the membrane (Moult et al, 2010). To determine at which area of the neuron GluR1 expression is increased, immunocytochemistry with synapsin-1 was completed and it was found that GluR1 and synapsin-1 colocalize, showing that GluR1 expression is increased at synapses (Moult et al, 2010). It was also shown that leptin receptors and NMDA

receptors were required for these effects. Collectively, these studies show that leptin increases glutamate receptor trafficking at the synapses of hippocampal neurons, which may be an important component of leptin-mediated induction of LTP.

The proteins growth-associated protein 43 (GAP-43) and synaptosomal-associated protein 25 (SNAP-25) are implicated in being involved in the maintenance phase of LTP and have been shown to be influenced by leptin. For example, phosphorylated GAP-43 is important in transitioning from LTP induction to LTP maintenance (Reymann et al, 1988; Leahy et al, 1993). SNAP-25 is also involved in LTP, as blocking SNAP-25 function resulted in blocking LTP (Roberts et al, 1998). These proteins are both located presynaptically and can be regulated by leptin (Ahima et al, 1999). When leptin is deficient or absent, SNAP-25 is decreased and GAP-43 is increased 3-fold. Both proteins return to normal levels once leptin is applied (Ahima et al, 1999). In a leptin deficient model, there are dysregulated/abnormal levels of SNAP-25 and GAP-43 signaling molecules. This shows the importance of leptin in synaptic proteins that have been directly linked to the maintenance phase of LTP and spatial memory (Ahima et al, 1999; Reymann et al, 1988; Leahy et al, 1993).

2.2 LEPTIN & MORPHOLOGY

Dendritic spines are projections on the dendritic shaft that can number in the thousands per dendrite and are a major site of excitatory input from a presynaptic neuron (Gray, 1959; Harris and Kater, 1994; Shepherd, 1996; Muller and Connor, 1991; Yuste and Denk, 1995; Yuste and Bonhoeffer, 2001). Spines are able to control how long transient calcium is present, an important aspect of synaptic plasticity (Lynch et al, 1983; Malenka et al, 1989). There are several types of shapes of dendritic spines: thin with a

small spine head and long spine neck, mushroom-shaped with a larger head and short neck, or stubby with only a small protrusion. The shape of the dendritic spines can impact synaptic transmission and the shape and number of spines have been linked to learning, memory, depression and anxiety (Pittenger and Duman, 2008; Penzes et al, 2011).

Dendritic filopodia are small protrusions on dendrites that can mature into dendritic spines. They are very motile and transient and synaptic transmission, particularly with glutamate, is critical in stabilizing filopodia (McKinney et al, 1999). Morphology changes are correlated to changes in synaptic strength (Maletic-Savatic et al, 1999; Toni et al, 1999; Yuste and Bonhoeffer, 2001). Morphology changes that occur post-LTP have been shown to involve larger spine heads and shorter spine necks which results in an increase in current into the spine and faster calcium storage in the spine (Yuste and Bonhoeffer, 2001).

Changes in dendritic spines of neurons in the hippocampus can be stimulated by a variety of factors, including hormones like estradiol and progesterone as well as other factors such as exercise and restricting calorie intake (Gould et al, 1990; Woolley et al, 1990; Stranahan et al, 2009). Specific changes to dendritic spines include spine shape, density and number and these morphological changes result in functional changes due to dendritic spines being a site of neurotransmission (Woolley et al, 1990). All of these aspects of dendritic morphology have been shown to change in response to leptin.

During development, leptin plays a role in axon projection growth from the arcuate nucleus of the hypothalamus to the paraventricular nucleus and *in vitro*, leptin has been shown to support the growth of neurites, any projection from a neuronal cell body (Bouret et al, 2004). Leptin can also increase neurite growth in the hypothalamus and

cerebellar purkinje neurons (Bouret et al, 2004; Oldreive et al, 2008). These data from outside the hippocampus further support the role of leptin in inducing morphology changes (Oldreive et al, 2008). Leptin increases spine density *in vivo* and *in vitro*, slices and cultures, respectively (Dhar et al, 2014; O'Malley et al, 2007). This is seen with synapsin-1 staining of the somata and neurites after leptin was administered (O'Malley et al, 2007). Studies in a model of leptin signaling deficiencies show that leptin receptor function is critical for normal dendritic spine density, as these animals had less dense spines than controls in areas CA1 and CA3 of the hippocampus (Dhar et al, 2014).

In order to assess whether leptin may effect dendritic spines that could lead to functional synapse formation, hippocampal cell cultures were used to show that leptin application increased spine density. Dendritic spines themselves can also be matured in the presence of leptin and a leptin-deficient state results in smaller spine density, supported by other studies in another model (O'Malley et al, 2007; Dhar et al, 2014). In the same set of experiments, it was determined that excitatory transmission is critical in these morphological changes, mediated through NMDA receptors but not AMPA receptors (O'Malley et al, 2007).

Dendritic filopodia have been shown to be affected by leptin application (O'Malley et al, 2007). In the presence of leptin, neurons saw a significant increase in dendritic filopodia number, or density (O'Malley et al, 2007). To determine whether or not leptin receptor activation was required for the increase in density, siRNA was used to reduce the expression of the leptin receptor. It was found that the density of the dendritic filopodia decreased as the leptin receptor expression decreased, implying that the leptin receptor was required for the increase in density of filopodia (O'Malley et al, 2007).

Leptin can also increase the formation of new extensions on the filopodia, as seen by the appearance of growth cones (O'Malley et al, 2007). Additionally, leptin can influence re-organization of actin protein cytoskeleton in neurons from the base of the dendrite to the filopodia, changes in the actin cytoskeleton being critical for any change in morphology (O'Malley et al, 2007; O'Malley et al, 2005).

Motility of the dendritic filopodia was also increased in the presence of leptin (O'Malley et al, 2007). Leptin exposure also resulted in the lengthening of neurites that were similar to filopodia. Although leptin has been shown to signal using the PI3K pathway for fast actions, any effects that leptin has on dendritic morphology were found to be mediated through MAPK signaling (O'Malley et al, 2007). It was suggested that STAT3 signaling was most likely not involved in the fast morphology changes seen during the experiments (O'Malley et al, 2007).

Morphology changes in dendritic spines and synaptogenesis, the generation of new synapses, are closely linked. The early stages of synaptogenesis are marked by motility of dendritic filopodia, and this has been proposed as being how new synapses are formed (Ziv and Smith, 1996). A study supports this by showing that the effect of leptin that results in increasing dendritic spine filopodia number and motility leads to the formation of new and active synapses (Dhar et al, 2014). Dendritic filopodia are involved in synaptogenesis such that they can initiate new synaptic contacts (Ziv and Smith, 1996). One study has shown that spine density and levels of synaptic proteins synapsin-1-positive puncta increase after exposure to leptin (O'Malley et al, 2007). This would support the idea that leptin can increase synaptic contacts and therefore synaptogenesis.

MAPK signaling is required for these morphology changes but not PI3K signaling (Harvey, 2013; O'Malley et al, 2007).

Leptin influences morphology in a similar manner to how LTP results in morphology changes (Maletic-Savatic et al, 1999). Spine density, filopodia number and motility increases both with leptin and after LTP occurs (Dhar et al, 2014; O'Malley et al, 2007). This morphological evidence shows that leptin can influence the dendritic spines and filopodia, a major site of excitatory input to a post-synaptic neuron which can alter the neuron's overall excitability in addition to promoting synaptogenesis and may be an important component of leptin-induced LTP (Maletic-Savatic et al, 1999).

2.2.1 NEUROGENESIS

Cell proliferation, the addition of new cells, and neurogenesis, the addition of new neurons, may also be considered a morphological change indicating synaptic plasticity. Before the birth of an organism, neurogenesis takes place in the ventricular zone and after birth, takes place in the subgranular zone (Schlessinger et al, 1975; Altman and Bayer, 1990; Schoenfeld and Gould, 2012; Leuner and Gould, 2010; Altman 1962; Altman and Das 1967; Kaplan and Hinds 1977; Gould et al, 1999b).

Many factors can effect neurogenesis and how long new neurons are able to survive, such as stress and psychotropic drugs like antidepressants (McEwen, 1999; Gould et al, 1997; Duman et al, 2001; Dranovsky and Hen, 2006). For example, acute stress decreases cell survival as compared to social defeat stress and chronic stress that can decrease cell proliferation, a different stage of neurogenesis (Gould et al, 1997; Gould et al, 1998; Schoenfeld and Gould, 2012; Yap et al, 2006). Other factors such as

aging and glucocorticoids decrease cell proliferation (Kuhn et al, 1996; Seki and Arai, 1995; Cameron and Gould, 1994; Cameron and McKay, 1999), while learning (Gould et al, 1999a), exercise and seizures (Parent et al, 1997) increase neurogenesis (van Praag et al, 1999). There are even sex differences, if rats are isolated after chronic shock, male rats show less survival of new neurons whereas female rats increase survival of new neurons (Westenbroek et al, 2004). Interestingly, exposure to a predator odor decreases cell proliferation in male rats but not female rats (Falconer and Galea, 2003). In addition to these factors, leptin can also influence neurogenesis.

The effects of leptin on all three stages of neurogenesis has been assessed and it has been found that leptin can influence both the cell proliferation and differentiation of progenitor cells (Garza et al, 2008). Adult mice were treated with different doses of leptin and injected with BrdU, a marker for proliferating cells. Progenitor cells were collected and analyzed for presence of the leptin receptor mRNA. After 2 weeks of treatment with leptin and BrdU, there was a significant increase of BrdU-labeled cells in the dentate gyrus of the hippocampus compared to vehicle-treated controls (Garza et al, 2008). This shows that leptin increases cell proliferation. After 28 days, to allow new cells to differentiate, the leptin-treated group still had significantly more BrdU-labeled cells than controls. Cells were double-labeled with antibodies against either NeuN and GFAP and BrdU to discern between new neurons and new glial cells, respectively. This revealed that in the leptin treated group, most cells were NeuN-positive, confirming the new cells were neurons, with only a small percentage colocalization with BrdU and GFAP. Interestingly, the proportion of double-labeled BrdU and NeuN cells vs GFAP-BrdU labeled cells did not differ between the vehicle-treated control group and leptin-treated

group. To show the effects of leptin on cell survival, animals were injected with BrdU pre-leptin treatment, however there was no difference between the number of surviving cells with the vehicle and leptin treated groups. Immunocytochemistry in cultured progenitor cells indicated that both the leptin receptor mRNA and protein were present (Garza et al, 2008). This data shows that leptin does not have a significant effect on cell differentiation or cell survival but instead enhances the first stage of neurogenesis, cell proliferation.

To further investigate the role of leptin in cell proliferation, studies were completed in adult hippocampal progenitor cultures. The effects of leptin on cell proliferation in culture was dose-dependent, a dose of 1nM resulted in the highest amount of BrdU-labeled cells and higher doses had no effects, reinforcing other studies that found an inverted U shape for maximum responses (Garza et al, 2008). These experiments in cultures to assess the effects of leptin on differentiation and survival showed similar results as the *in vivo* studies as no differences were found between leptin and control groups (Garza et al, 2008). In determining which signaling pathways leptin activated in progenitor cells in culture, it was found that phosphorylation of Akt of the PI3K pathway and STAT3 of the JAK2/STAT3 pathway were increased after 15 minutes of treatment with leptin (Garza et al, 2008). There was no significant change in the phosphorylation of ERK after leptin treatment, indicating that leptin activated JAK2/STAT3 and PI3K signaling but not MAPK signaling (Garza et al, 2008). Blocking Akt and STAT3 with an inhibitor resulted in significantly less BrdU-labeled cells treated with leptin, reinforcing the data that leptin utilizes PI3K and JAK2/STAT3 for signaling in progenitor cells of the dentate gyrus in the hippocampus (Garza et al, 2008).

Leptin not only affects cell proliferation but has also been shown to have a rescue-effect on neurogenesis after a chronic unpredictable stress (CUS) paradigm. Stress has been shown to substantially decrease neurogenesis in the dentate gyrus and CUS has been shown to cause depressive-like symptoms in animals (Jayatissa et al, 2006). Glucocorticoids can also decrease neurogenesis while antidepressant medications increase neurogenesis (Dranovsky and Hen, 2006). A series of experiments subjecting rats to chronic unpredictable stress for 21 days revealed deficits in neurogenesis (Garza et al, 2013). BrdU labeling after stress revealed that CUS had a reducing effect on the number of BrdU labeled cells in the dentate gyrus (Garza et al, 2013). Leptin was then administered to CUS animals for 2 weeks after the entire 21-day CUS paradigm. Neurogenesis was analyzed by counting BrdU-labeled cells in the dentate gyrus and it was found that leptin treatment significantly increased the amount of BrdU-labeled cells when administered chronically (Garza et al, 2013). The new cells were mostly neurons, as seen by double-labeling with NeuN and BrdU (Garza et al, 2013). Cell survival was analyzed 28 days later and the number of BrdU-labeled cells that survived with leptin treatment was more than with CUS stress alone; and CUS animals showed lower numbers of surviving cells than the non-CUS and non-leptin treated groups (Garza et al, 2013). Body weight was decreased in all groups subjected to CUS and leptin treatment groups saw additional body weight reductions, a normal result of stress and leptin treatment (Garza et al, 2013). This data has shown that leptin can almost completely reverse the deficits in neurogenesis after a CUS model.

It has been shown that rats subjected to chronic stress show anhedonia and a decrease in dentate gyrus neurogenesis (Jayatissa et al, 2006). The depressive-like

symptoms have been alleviated by the antidepressant escitalopram, administered systemically via an intraperitoneal (i.p.) injection, and neurogenesis was restored (Jayatissa et al, 2006). Antidepressants seem to inhibit the stress effects (Dranovsky and Hen, 2006). This data mirror the effect of leptin on neurogenesis, as demonstrated by an experiment with chronic unpredictable stress and leptin treatment. This suggests that leptin has an antidepressant-like effect possibly involving the restoration of neurogenesis (Jayatissa et al, 2006; Garza et al, 2013).

Neurogenesis is an important aspect of synaptic plasticity because new neurons in the dentate gyrus have been shown to produce LTP more readily than more mature neurons (Snyder et al, 2001). Under normal physiological conditions, granule neurons have inhibitory GABAergic inputs to control their excitation (Snyder et al, 2001). It was proposed that new neurons had much less GABAergic input that would inhibit them, making activation of these neurons easier. A study has shown the presence of two different types of LTP in the medial perforant pathway, one type presumably from new neurons and another from old neurons (Snyder et al, 2001). The first, small current LTP was induced in hippocampal slices in artificial cerebrospinal fluid (ASCF) after tetanus whereas a second, much larger LTP occurred after picrotoxin was added (Snyder et al, 2001). Additionally, the small LTP was blocked by ifenprodil, an NR2B NMDA receptor subtype antagonist in contrast to the larger LTP that was not blocked by ifenprodil. These results indicate two sets of neuron populations that react differently and have different amplitudes of LTP, not just two different synapses due to the GABAergic input. This study shows the importance of neurogenesis and how new neurons may play a role in learning (Moser, 1996).

Collectively, leptin has a clear enhancement of cell proliferation both *in vivo* and in cultures and signals with both the JAK/STAT and PI3K signaling pathways for these effects (Garza et al, 2008). Leptin also has a rescue effect of the reduction of neurogenesis in a model of chronic unpredictable stress and has striking neurogenesis-restoring abilities similar to the effects of an antidepressant (Garza et al, 2013). In addition to these effects on neurogenesis, leptin can also alter behavior.

2.3 LEPTIN & BEHAVIOR

Behavioral tests allow for the study of overall effect on function as a result of some stimulus, such as leptin. There are many behavioral tests to assess specific types of learning and memory, for example the Morris Water Maze for spatial learning and memory, the novel object recognition test to assess hippocampal-dependent memory and passive shock avoidance to test emotional learning and memory. The Morris Water Maze is a hippocampal-dependent, spatial learning and memory test. The test consists of a circular tank of water with four quadrants and an opaque, submerged platform in one quadrant. The platform remains in the same quadrant for the duration of the test. Training consists of placing the animal in the water and the animal swims until it finds the platform. Swimming time, distance and speed are all measured. On test day, the platform is removed and the time and distance taken to find the location where the platform was, the goal area, is measured. Shorter swimming times and distances indicate better performance and speed is measured to determine whether or not all groups of animals are operating with the same locomotor activity (Oomura et al, 2006).

The novel object recognition tests for hippocampal-dependent working memory performance with a new object and old object. The animal is placed in a chamber or box and is allowed to explore or habituate a day before testing. Training consists of the chamber containing of two of the same novel objects and the animal is allowed to explore for a certain amount of time, for example 10 minutes. Next, the animal is reintroduced to the same chamber and with two objects, one being a previously explored object and one being entirely new. The following aspects are analyzed: time and frequency spent exploring each object as well as other behaviors such as grooming and rearing. The discrimination index measures recognition memory: total time spent with new object/total time of object exploration (Greco et al, 2010). More time spent with the new object indicates better performance in terms of working memory.

The passive avoidance task consists of a box with light and dark sections separated by a guillotine door. The animal is placed in the light section for some amount of time then the door opens and the animal is allowed to move to the dark section. After this occurs, the door is closed and a footshock is delivered. Latency, the delay in entering the dark section, is measured and a longer latency is indicative of a better performance in emotional learning (Oomura et al, 2006).

Leptin treatment has been shown to elicit differences compared to controls in many behavioral tests. Leptin injections directly into the hippocampus improves spatial and emotional learning, memory and retention as determined by several behavioral analyses (Harvey, 2007; Farr et al, 2006; Oomura et al, 2006). For example, leptin improves performance in the passive shock avoidance and MWM behavioral tests dose-dependently (Oomura et al, 2006). Different doses of leptin result in improved memory

performances again in an inverted U with the lowest and highest doses of leptin had longer swimming times, swimming distances, and less time spent in the area of the platform. More time spent in the area where the hidden platform was previously, shorter swimming distances and times indicate better memory performance. This dose-dependent effect is similar to what was seen with leptin in other experiments with leptin having dose-dependent effects on LTP, CaMKII activity and cell proliferation (Garza et al, 2008; Oomura et al, 2006; Wayner et al, 2004).

The passive avoidance shock test, designed to test emotional learning, showed an inverted-U dose response to leptin. Using the same doses as in the Morris Water Maze, middle doses of leptin, 5 u g/kg and 50 u g/kg, resulted in longer latencies to step down, indicative of better performance (Oomura et al, 2006). These behavioral differences were unrelated to changes in locomotion or changes in pain sensitivity, as behavioral response to a hot-plate was similar among all groups.

Another study found similar findings, i.p. injected leptin resulted in significant increased performance in the Morris Water Maze compared to vehicle-injected controls (Rasi et al, 2007). In addition, medium doses had the most significant increase in spatial memory, a consistent finding between several groups (Rasi et al, 2007; Oomura et al, 2006; Garza et al, 2008). Possible reasons behind the dose-dependent effect of leptin increasing spatial learning and memory as well as cell proliferation in neurogenesis may be related to the leptin-activated potassium channel (Spanswick et al, 1997; Shanley et al, 2002). This channel opens after prolonged excitation and may open with higher doses of leptin, hyperpolarizing the membrane. It is also possible that low doses of leptin may not be effective due to the dose being too small for effects to be seen.

To further support leptin improving behavioral testing performance, improvements have also been seen in the T-maze footshock avoidance and the one trial step down inhibitory avoidance test for memory in mice after leptin was administered bilaterally into the hippocampus (Farr et al, 2006). In outbred, CD-1 rats leptin administration before testing was shown to enhance performance in the T-maze footshock avoidance and the one trial step down inhibitory avoidance tests to assess memory processing. In contrast, if leptin was given after training, memory retention was not improved. In year-old SAMP8 mice, a mouse model of Alzheimer's Disease, intrahippocampal injections of leptin improved performance in the T-maze footshock avoidance test (Farr et al, 2006). Similar results were also found in year-old SAMP8 mice in the step down inhibitory avoidance test, where a longer latency to step down is indicative of a better performance.

In a series of experiments subjecting rats to chronic unpredictable stress for 21 days paired with the open field test and sucrose preference test revealed several deficits (Garza et al, 2013). Rats subjected to the CUS paradigm for 21 days had less exploratory behaviors in open field, anhedonia behaviors in sucrose preference and more immobility time in forced swim (Garza et al, 2013). These results indicate depressive-like characteristics. Leptin was then administered to CUS animals for 2 weeks after the entire 21-day CUS paradigm. Although there was no change in exploratory behaviors in open field, leptin reversed the anhedonia seen in the sucrose preference test in CUS animals and leptin treatment decreased immobility time, an indicator of depressive-like behaviors (Garza et al, 2013). Body weight was decreased in all groups subjected to CUS and leptin treatment groups saw additional body weight reductions (Garza et al, 2013). This

suggests that leptin has an antidepressant-like effect in reversal of depressive-like symptoms like anhedonia and immobility time in the forced swim test (Jayatissa et al, 2006; Garza et al, 2013).

In addition to the studies described above, models of leptin resistance or deficiency have also provided insight into the role of leptin in relation to the regulation of hippocampal synaptic plasticity. Experimental models of disrupted leptin signaling exhibit deficits in hippocampal synaptic plasticity thereby providing further evidence for a key role of leptin in mediating structural and functional activities of the hippocampus.

2.4 ANIMAL MODELS OF LEPTIN DYSFUNCTION

There are several models of leptin resistance or deficiency to demonstrate a dysregulated leptin state and the resulting changes in synaptic plasticity. One model is the Zucker rat which has a point mutation in its leptin receptors that results in impairments in signaling (Zucker and Zucker, 1961; Clark et al, 1983). The *db/db* mouse has malformed leptin receptor transcript which shortens the intracellular domain used for signaling, resulting in the inability to form a working leptin receptor (Chen et al, 1996; Chua et al, 1996). In both the Zucker rat and *db/db* mouse, leptin is still synthesized but normal signaling with the leptin receptor cannot occur (Li et al, 2002). In *db/db* animals, there is no change in mRNA expression for the microvessel BBB short isoform ObRa used for leptin transport and *db/db* mice show normal transport of leptin across the BBB (Hileman et al, 2002). The *ob/ob* mouse is leptin deficient through genetic modification but has normally functioning leptin receptors (Hummel et al, 1966). The diet-induced obese (DIO) animal model is fed a high-fat diet to achieve metabolic parameters associated

with obesity. The obesity and hyperphagic phenotype of the Zucker rat, *db/db* and *ob/ob* mice also appears with lesioning of the hypothalamus, demonstrating the importance of the hypothalamus in regulation of feeding and energy expenditure (Baylis et al, 1996). These models also give evidence that leptin also has a role in reproduction because *ob/ob* and *db/db* mice both have abnormalities with puberty, fertility and lactation (Sainsbury et al, 2002c; Donato et al, 2011).

The physiology of each of these models includes: obesity, leptin resistance or leptin deficiency, insulin resistance, increased body fat, and dyslipidemia, and endocrine dysfunction (Zucker and Antoniades, 1972; Pelleymounter et al, 1995; Halaas et al, 1995; Coleman et al, 1978; Ahima et al, 1996). These models demonstrate the importance of leptin in metabolic functions including glucose homeostasis, as administering leptin to the brains of *ob/ob* mice corrects glucose imbalances inherent in the *ob/ob* model (Asilmaz et al, 2004). And importantly, decreasing body weight does not entirely fix the glucose imbalances in these animals, showing that diet-induced obesity and obesity itself are not the only cause of the metabolic imbalances (Flak and Myers, 2015; Pelleymounter et al, 1995; Wyse et al, 1970; Schwartz et al, 1996). For these reasons, the Zucker rat, *db/db* mice *ob/ob* mice, and DIO rodents are often used as models of obesity, metabolic syndrome and type 2 diabetes mellitus.

2.5 ANIMAL MODELS AND SYNAPTIC PLASTICITY DEFICITS

Deficient leptin function, whether through the receptor or the circulating peptide itself, has several effects on synaptic plasticity in the hippocampus. Due to the fact that Zucker rats and *db/db* mice both have impairments in the leptin receptor, they both show

very similar synaptic plasticity impairments. One study investigated the differences between LTP, LTD and CaMKII activity Zucker rats, *db/db* mice and lean controls. It was found that hippocampal slices from fatty Zucker rats exhibited a lack of LTP in area CA3 with and without bath application of leptin and this LTP is normally evoked in control Zucker rats (Li et al, 2002). Experiments determining LTD in fatty Zucker rats showed similar impairments; fatty Zucker rats did not elicit LTD with or without leptin in a dose that normally would elicit LTD in controls. GABA and Quis were applied to slices and corresponding hyperpolarization was seen, however leptin application had no effect, showing that fatty Zucker rats were not responding to leptin normally (Li et al, 2002). Hippocampal slices from *db/db* mice were assessed for LTP and LTD qualities and it was found that *db/db* mice showed an inability to maintain LTP with or without leptin in solution. LTD was unable to be evoked and just as with Zucker rats, *db/db* mice have diminished LTP and LTD compared to controls. Calcium-independent CaMKII activity was assayed in both Zucker rats and *db/db* mice. Fatty Zucker rats showed significantly less CaMKII activity while *db/db* mice had no difference compared to controls (Li et al, 2002). Mechanistically, this may indicate deficits in the enzyme that could be causing the impairments in LTP and LTD. Other studies show impairments in the ability of fatty Zucker rats to exhibit normal LTP (Moult et al, 2010; Gerges et al, 2003).

Morphological abnormalities are also found in animal models with dysfunctional leptin. Density of excitatory and inhibitory synapses in *ob/ob* mice differ from control mice and interestingly, systemic leptin administration will normalize these changes in density in approximately 6 hours (Harvey, 2013; Pinto et al, 2004). Both *db/db* and *ob/ob* animals have been shown to have smaller brain weights, demonstrating additional

structure deficits (Ahima et al, 1999). Both animal models also have immature synaptic proteins and decreased myelin as well as fewer synaptic markers such as SNAP-25, syntaxin-1, and GAP, implying fewer synapses (Ahima et al, 1999). Administration of leptin to the *ob/ob* and *db/db* animals resulted in an increase in brain weight and corrected the reductions in synaptic-associated proteins SNAP-25, syntaxin-1 and GAP levels (Ahima et al, 1999).

Studies in *db/db* mice show that leptin receptor function is critical for normal dendritic spine density, as *db/db* mice had less spine density than controls in areas CA1 and CA3 of the hippocampus (Dhar et al, 2014). To further support this, *db/db* mice have been shown to have decreases in spine density in the dentate gyrus hippocampus *in vivo* and DIO models have smaller dendritic spine densities in area CA1 (Stranahan et al, 2009; Stranahan et al, 2008c). DIO mice also show impaired transport of leptin across the BBB compared to lean controls (Banks et al, 2003). This has been shown to be an decreased ability of the BBB to transport leptin, not just saturation of the leptin transport system (Banks et al, 2003). In lentivirus-mediated insulin receptor downregulated (IRAS) rats that exhibit an obese phenotype, a presynaptic protein and postsynaptic protein, synaptophysin and PSD-95 respectively, were found to have less organized PSD-95 and clustered SYN protein, implying a structural change (Grillo et al, 2011). These studies show the effect of leptin on brain weight, synaptic proteins and myelination and gives evidence that leptin is involved in brain structure and morphology.

Fatty Zucker rats have been shown to show less neuronal differentiation, as opposed to glial cell differentiation, part of the stages of neurogenesis and both fatty Zucker rats and *db/db* mice show less cell proliferation in the hippocampus (Yi et al,

2009). DIO rodents also show impairments in neurogenesis, specifically in cell survival (Lindqvist et al, 2006).

Behavioral analyses of Zucker rats and *db/db* mice have also shown that these animals have learning and memory impairments. Fatty Zucker rats had longer swimming distances and had less passes through the platform area in the probe test section of the Morris Water Maze compared to their lean counterparts (Li et al, 2002). Swimming speeds were no different between groups, indicating that Zucker rats were able to swim normally. The *db/db* mice were also subjected to the Morris Water Maze and there were differences in swimming distances between groups at the fourth block and *db/db* mice had less passes through the platform area. The groups had similar swimming speeds, indicating swimming ability was similar between groups. These behavioral data indicates that both Zucker rats and *db/db* mice have a decrease in spatial memory.

Another behavioral assessment, the go/no-go variable interval delayed alternation task (VIDA) has shown additional cognitive impairments in fatty Zucker rats. In the VIDA test, animals are placed in a Skinner box that contains a lever that, when pressed, allows food to enter a feeder next to it. Once animals were food restricted, they were trained to lever press for food. The VIDA test had trials that gave food when the lever was pressed (go trials) alternating with trials that did not give food (no-go trials). The time between each trial, the intertrial interval, would vary from 0 to 80 seconds. The fatty Zucker rats and lean Zucker rats had no differences in training with the lever or in learning the alternation test when there was 0 seconds of delay between go and no-go trials (Winocur et al, 2005). Latency to first leverpress was assessed in order to insure that each group could physically do the task. As the intertrial interval increased in time,

both groups showed a decline in ability however fatty Zucker rats had significantly worse performance than lean Zucker rats, indicating memory deficits at the longer intertrial interval as shown by the shorter latency on no-go trials. This deficit indicates an impairment of hippocampal-dependent memory and not a lack of motivation or ability (Winocur et al, 2005).

In a study with DIO rats that showed impairments in performance in the Morris Water Maze, it was found that an 8-week exercise intervention improved cognitive performance (Cheng et al, 2016). The IRAS rat model, in addition to being obese are also hyperleptinemic and hypertriglyceridemic and have shown hippocampal-dependent behavioral deficits (Grillo et al, 2011). The IRAS rats had deficits in contextually conditioned freezing, specifically less freezing behavior during retention, compared to controls (Grillo et al, 2011).

These studies in animal models of abnormal leptin have shown deficiencies in LTP, LTD, CaMKII activity, neurogenesis, morphology as well as learning and memory. In addition to these metabolic disorders, animal models of leptin dysfunction also show depressive and anxiety-like deficits, demonstrating that leptin plays a role in cognitive disorders.

2.6 LEPTIN IN DEPRESSION, ANXIETY & STRESS

In addition to electrophysiological, morphological and hippocampal-dependent memory deficits, as seen by the VIDA and Morris Water Maze tests, *db/db* mice show cognitive impairments and depressive-like behaviors (Sharma et al, 2010; Moult et al, 2010; Gerges et al, 2003; Ahima et al, 1999; Winocur et al, 2005; Li et al, 2002).

Anxiety-like behaviors are seen in some studies and not in others, although this may be due to different behavioral testing, species or strains (Sharma et al, 2010; Liu et al, 2010).

Both young and adult *db/db* mice show impairments in the forced swim test, a behavioral test for depressive-like behaviors (Sharma et al, 2010). In a study investigating depressive-like and anxiety-like features in young and older *db/db* mice, control mice and *db/db* mice were separated into groups of juvenile 5-6 weeks old and adult, 10-11 weeks old (Sharma et al, 2010). Extensive behavioral testing was completed, including the forced swim test, pre-pulse inhibition test, elevated plus maze, open field and Y-maze test. The pre-pulse inhibition test consists of mice being placed in startle chambers followed by a white noise that is sounded five times and is designed to measure psychosis-like behavior. The Y-maze tests working memory by using exploratory behavior, mice placed in intersection of Y and tested to see how many times the mouse entered an arm, entering into 3 different arms after each other referred to as an alternation.

In the forced swim test, both young and old *db/db* mice showed longer immobility times, indicating behavioral despair analogous to a depressive-like behavior (Sharma et al, 2010). During the pre-pulse inhibition, normal startling was seen in young animals but not old, adult *db/db* mice had lower percent pre-pulse inhibition (PPI) compared to lean controls. In the elevated plus maze, same distance was traveled in the open arms of the maze by *db/db* mice compared to controls but juvenile and adult *db/db* mice spent significantly more time in the open arms, indicating an anti-anxiety-like behavior (Sharma et al, 2010). In the open field test, both groups of *db/db* mice showed less activity counts for basic and fine movement than controls but all groups spent more time

exploring the periphery of the open field instead of the center area, with no difference between *db/db* and controls in proportion of time spent in the periphery versus the center (Sharma et al, 2010). The Y-maze test showed that adult *db/db* mice had less arm entries and alternations but no change in percent Y-maze scores, showing no significant difference in working memory between adult *db/db* mice and age-matched controls. These data show that in many different behavioral tests, adult *db/db* mice in particular show depressive-like and psychosis-like behaviors although *db/db* mice showed less anxiety-like behaviors than lean controls with no differences in working memory between groups (Sharma et al, 2010).

Additional studies looking at depressive-like and anxiety-like behaviors were completed to focus on the role of the hippocampal leptin receptors in these behavioral states. Deletion of leptin receptors in the hippocampus, using the targeted Cre/lox system, resulted in anhedonia in the sucrose preference test and depressive-like behaviors in the tail suspension test (Guo et al, 2012). Leptin or vehicle was administered via an i.p. injection to leptin receptor knockout mice and wild type mice and then the forced swim or tail suspension tests were conducted. Leptin receptor knockouts treated with saline showed more immobility time than controls and leptin significantly decreased immobility time in wild type mice while having no effect on leptin receptor knockout mice. Additionally, the sucrose preference test showed leptin knockouts had a much lower preference for the sucrose solution than wild-type controls (Guo et al, 2012). Anxiety-like behaviors were also analyzed using the elevated plus maze and open field tests however there were no differences between leptin receptor knockouts and controls, similar results to another study looking at anxiety-like behaviors in *db/db* mice (Guo et al, 2012; Sharma

et al, 2010). These data shows that leptin receptor knockout mice exclusively in the hippocampus were unable to have their depressive-like behavior reversed when leptin was administrated while leptin resulted in less depressive-like behavior in wild type mice, indicating that leptin can result in antidepressant effects that are mediated through hippocampal glutamatergic neurons.

In a study looking at the effects of leptin and fluoxetine administration on several tests for anxiety-like behavior, it was found that leptin is anxiolytic (Liu et al, 2010). Male C57BL/6J mice, with normal leptin and leptin receptor function, were given either i.p. leptin or fluoxetine injections and then subjected to the tail suspension and forced swim tests for depressive-like behavior. It was found that both leptin and fluoxetine significantly decreased immobility time compared to vehicle controls, immobility as an indicator of behavioral despair or depressive-like symptoms. Anxiety-like behaviors were assessed in a similar fashion, using the elevated plus maze. It was found that following an i.p. leptin injection, leptin administration resulted in increased open arm time and the number of entries into the open arms (Liu et al, 2010). Both time in the open arms and number of open arm entries indicate an anti-anxiety-like effect. Interestingly, the lower dose of leptin did not result in these anxiolytic effects and fluoxetine decreased open arm entries and open arm time. The social interaction test was also completed to assess social behavior and leptin injection resulted in an increase in the total social interaction time, compared to controls (Liu et al, 2010). Additionally, leptin injection resulted in less time spent grooming, a function that can indicate anxiety following a stressful event. Interestingly, neither leptin nor fluoxetine resulted in any significant anxiolytic reaction in the open field test, similar to other studies that have found no significant effect of

leptin in the open field test (Liu et al, 2010; Guo et al, 2012; Sharma et al, 2010). This may imply that leptin is able to have anxiolytic effects in certain tests of anxiety and so perhaps leptin may play a role in particular types of anxiety. These data also collectively show how leptin even plays a role in social interaction and social anxiety.

In contrast to studies showing that depressive-like behaviors in *ob/ob* mice or normal animals can be alleviated by leptin administration, diet-induced leptin resistant models show the opposite. Depressive-like behaviors in diet-induced obese (DIO) mice and control-diet mice given a leptin injection were analyzed with the forced swim test. After the leptin injection, control diet animals had a lower immobility time, indicating less depressive-like behaviors (Yamada et al, 2011). Conversely, in the DIO mice, immobility time was longer compared to controls and there was no difference after leptin injection. In *ob/ob* mice, however, leptin injection decreased immobility time indicating less behavioral despair and an antidepressant-like effect of leptin (Yamada et al, 2011). DIO mice showed not only more depressive-like behaviors but also no response to the antidepressant-like effects of leptin. There was also no change in locomotor activity, indicating that body mass had no effect on swimming (Yamada et al, 2011).

Another test of depressive-like behaviors, the sucrose preference test, showed that DIO mice had a much lower preference for sucrose water than controls (Yamada et al, 2011). Levels of *c-Fos*, a marker of neuronal activity, were determined in the hippocampus after leptin injection and it was found that in control-diet mice, *c-Fos* immunoreactive cells increased significantly post-leptin injection, indicating an increase in activity in this area but no such activation was seen in DIO mice (Yamada et al, 2011). Collectively, this data show that diet-induced obese mice and *ob/ob* mice show

depressive-like behaviors and leptin has an antidepressant-like effect in control-diet mice and *ob/ob* mice but not DIO mice. DIO mice do not have any genetic modifications and therefore could have a more complicated dysregulated metabolic system that could explain the differences in leptin treatment between DIO mice and *ob/ob* mice.

Leptin administration has been shown to play a role in stress. Chronic unpredictable stress and chronic social defeat are two models that have been shown to produce depressive-like behaviors in rats (Lu et al, 2005). The role of leptin in the alleviation of depressive-like symptoms after CUS and chronic social defeat were assessed. Depressive-like symptoms were measured using the forced swim test for despair and sucrose preference. CUS resulted in lower than control plasma leptin levels and higher corticosterone levels (Lu et al, 2005). Peripheral leptin administration corrected deficiencies in sucrose preference test after CUS and interestingly, leptin administration to non-stressed rats had no effect on the sucrose preference outcome (Lu et al, 2005). In the forced swim test, leptin administration resulted in a decrease in immobility time and an increase in swimming time, immobility time indicating behavioral despair, or a depressive-like phenotype (Lu et al, 2005). No effect of leptin on corticosterone was seen, indicating that leptin was not affecting the stress response. Amount of Fos immunoreactive neurons were measured in areas CA1, CA3 and the dentate gyrus and more activation was seen in leptin animals than saline controls (Lu et al, 2006). It is suggested that the hippocampus may then be a target for antidepressant-like effects by leptin (Lu et al, 2006).

To further assess the idea that the hippocampus is an area for antidepressant-like effects, leptin was injected directly into the hippocampus. Leptin administration resulted

in decreased immobility time and increased swimming in the forced swim test, very similar to the results of systemic leptin administration (Lu et al, 2006; Liu et al, 2010). To see whether the hypothalamus would have similar effects as injection into the hippocampus, leptin was directly injected into the hypothalamus. However, no effects on the forced swim test immobility time or swimming time were seen, as compared to control and only lower body weights were seen after 1-2 days (Lu et al, 2002). This would imply that the hippocampus is critical in mediating the ability of leptin to result in antidepressant-like effects. This comprehensive study shows that leptin plays a critical role in the hippocampus in alleviating depressive-like behaviors, as seen by the the sucrose preference and forced swim tests. Circulating plasma leptin was shown to decrease during chronic stress and was accompanied by depressive-like behaviors. The direct injection or indirect systemic injection of leptin normalized these depressive-like behaviors and this appears to have been mediated by the hippocampus (Liu et al, 2010; Lu et al, 2006).

Leptin, as previously discussed, increases neurogenesis in the hippocampus and antidepressants have also been shown to increase neurogenesis (Garza et al, 2008; Garza et al, 2013; Dranovsky and Hen, 2006). Chronic unpredictable stress decreases neurogenesis and also results in depressive-like behaviors that can be corrected by leptin (Lu et al, 2005; Lu et al, 2006; Jayatissa et al, 2006). This may indicate a link between leptin, neurogenesis and depression and perhaps some the antidepressant-like effects of leptin are mediated by the hippocampus, as seen by the *c-Fos* studies for neuronal activation (Lu et al, 2006; Yamada et al, 2011).

Studies done in animal models looking at the relationship between leptin and depression also may be applicable to humans. Major depressive disorder correlates with low leptin levels (Jow et al, 2006; Kraus et al, 2001). One study found an inverse relationship between scores on tests for depression, the Hamilton Rating Scales for Depression (HAM-D) and Perceived Stress Scale (Lawson et al, 2012). The higher the score on these evaluations of depression, the lower the leptin levels. Conversely, schizophrenia is associated with high levels of leptin, however this could be a result of side effects from antipsychotic medications taken for schizophrenia (Jow et al, 2006).

In addition to leptin playing critical roles in depression, anxiety and stress, leptin has also been shown to be involved in cognitive, learning and memory deficits in dementia and Alzheimer's disease which further support leptin having an important role in synaptic plasticity.

2.7 LEPTIN & ALZHEIMER'S DISEASE

To support the role of leptin in cognition, learning and memory, studies in various models of Alzheimer's Disease have shown improvements in memory function with leptin administration as well as a correlation between circulating levels of leptin and the disease. In addition, it has been found that high levels of leptin are protective against the symptoms of Alzheimer's disease.

Leptin levels have been shown to be dysregulated in aged rats and in mouse models of Alzheimer's disease. In humans, plasma leptin levels correlate with the progression of the disease, in that higher leptin levels correlated with less cognitive decline over a longitudinal study of four years (Holden et al, 2009). Aged rats are less

responsive to leptin, compared to young controls, as demonstrated by an experiment showing how leptin infused into young and old rats show differences percent decrease in food intake (Scarpace et al, 2000). Young rats showed a 50% decrease while old rats only showed 20%, showing that the normal effects of leptin on decreasing food intake mediated by the hypothalamus were not as effective in older rats (Scarpace et al, 2000). A decreased response to leptin in the hypothalamus in this experiment resulted in deficits in feeding behaviors which could possibly translate to a decreased response to leptin in the hippocampus with corresponding learning and memory deficits. This shows how leptin levels are abnormal in Alzheimer's Disease and aging and therefore provide a basis to analysis the roles of leptin in cognition, learning and memory.

The SAMP8 mouse model of Alzheimer's disease has learning and memory deficits due to an overproduction of the amyloid precursor protein and amyloid β protein, accumulation of amyloid β protein into amyloid plaques being one of the major hallmarks of the disease (Morley et al, 2000). Blocking the amyloid β precursor protein has resulted in memory and memory retention function improvements (Morley et al, 2000; Banks et al, 2001). Antibodies to β amyloid have similar increases in memory retention and acquisition (Morley et al, 2000). Interestingly, leptin has been shown to play a role in blocking amyloid β and can reverse amyloid β-induced LTP and glutamate receptor trafficking inhibition (Doherty et al, 2013). The SAMP8 mouse model accumulates amyloid β protein in many areas of the brain, in areas including the cingulate cortex, septum and hippocampus (Morley et al, 2000). Amyloid β has been shown to inhibit LTP induction and facilitate the reabsorption of glutamate receptors from the membrane in the hippocampus (Irving and Harvey, 2014). These animal models have shown deficits in

hippocampal-dependent learning (Farr et al, 2006). Both SAMP8 mice and CD-1 control mice were administered leptin and then subjected to the T-maze and step down inhibitory avoidance. SAMP8 mice given leptin saw improvements in both tests and interestingly, the older SAMP8 mice showed improvements at a lower dose of leptin than mice at 4 months of age who also had less amyloid β (Farr et al, 2006). This strengthens evidence that leptin is able to improve memory performance, even in a model with memory deficits.

The other hallmark of Alzheimer's Disease is the presence of neurofibrillary tangles that are a result of the excessive phosphorylation of tau protein (Beccano-Kelly and Harvey, 2012). Leptin can inhibit GSK3β activity and dose-dependently reduce tau phosphorylation (Freude et al, 2005; Valerio et al, 2006; Platt et al, 2016). It has been suggested that leptin interferes with the β amyloid production enzyme, β secretase, to reduce amyloid β (Beccano-Kelly and Harvey, 2011; Greco et al, 2008). Leptin could also act on the ApoE enzyme that then induces uptake β amyloid into neurons for degradation (Greco et al, 2008). Chronic leptin treatment resulted in a reduction of levels of amyloid β and phosphorylated tau in experiments in CRND8 mice, a model that has memory deficits and an Alzheimer's disease-like pathology (Greco et al, 2010). This is supported by experiments in neuronal cells treated with leptin, as leptin treatment reduced tau phosphorylation and reduce amyloid β (Greco et al, 2008).

The CRND8 mouse model of Alzheimer's Disease has also shown improvements in hippocampal-dependent memory tests after leptin treatment. Two memory tests were used, the novel object recognition test and trace fear conditioning, novel object for new object vs old object exploration to test recognition memory and trace fear conditioning

for ability to recall an negative stimulus and relate it to a particular environment (Greco et al, 2010). These hippocampal-dependent memory tests showed that leptin treated CRND8 mice performed almost identically to wild type mice whereas CRND8 mice treated with saline performed significantly worse (Greco et al, 2010). This shows that the CRND8 mice treated with chronic leptin were able to improve their memory performance equal to that of wild-type mice (Greco et al, 2010).

Plasma leptin levels are determined by eating and adiposity in that leptin increases after eating and leptin levels increase with increased adiposity (Zhang et al, 1994; Maffei et al, 1995). As adiposity increases in the form of obesity, leptin resistance begins to develop where leptin levels are high but signaling is not functioning normally to decrease feeding and increase energy expenditure. Obesity and lack of exercise have been linked to the development of Alzheimer's disease and low plasma leptin levels are also correlated to an increased chance of developing dementia (Beccano-Kelly and Harvey, 2011; Lieb et al, 2009). Higher levels of plasma leptin were found to correlate with increased volume of the hippocampus and are protective against Alzheimer's disease development (Lieb et al, 2009; Narita et al, 2009; Holden et al, 2009).

2.7.1 LEPTIN & NEUROPROTECTION

In addition to being protective against the development of Alzheimer's disease, other functions of leptin include more general neuroprotection and protection against apoptosis. Leptin can protect against oxidative stress under pathological conditions, for example with traumatic and ischemic brain injury (Zhang et al, 2008; Zhang et al, 2012). Leptin can also protect against excitotoxicity that can damage or kill neurons, as well as

protect against dementia, stroke and neuropathy (Dicou et al, 2001; Zhang et al, 2008; Guo et al, 2008; Lieb et al, 2009).

Leptin has antiapoptotic effects as well, cell cultures of a human neuroblastoma cell line were used to study these effects and it was found that leptin application increases cell number (Russo et al, 2004). The signaling pathways involved in the antiapoptotic effects of leptin were found to involve JAK/STAT, MAPK and PI3K with the ObRb (Russo et al, 2004). Leptin also has been shown to suppress apoptosis in non-neuronal cells, for example matured neutrophils and T cells, and the PI3K and MAPK pathways were found to be utilized (Bruno et al, 2005; Russo et al, 2004). These antiapoptotic effects are similar to the effects of leptin on progenitor cells that eventually differentiate into neurons, in that leptin can increase their number (Garza et al, 2008). Similarly, leptin can also increase overall hippocampal volume (Perez-Gonzalez et al, 2011).

2.7.2. LEPTIN AS A TREATMENT

There is mounting evidence that shows leptin administration has positive and significant effects on cognition, learning and memory. Leptin can improve some of the memory deficiencies in Alzheimer's disease as well as decrease tau phosphorylation (Freude et al, 2005; Valerio et al, 2006; Platt et al, 2016; Greco et al, 2008; Greco et al, 2010). In normal mice, leptin has been shown to improve learning and memory in several behavioral tests (Rasi et al, 2006) and enhance LTP, dendritic spine density and neurogenesis (Wayner et al, 2004; Shanley et al, 2001; Garza et al, 2008; O'Malley et al, 2007). Humans with congenital leptin deficiency have shown increases gray matter in some areas including the hippocampus after leptin replacement therapy (Paz-Filho et al,

2008; Matochik et al, 2005). Developmental delays in cognition, obesity, hypertension and dyslipidemia were all improved after two years of recombinant methionyl human leptin treatment in a five-year-old male patient with a leptin gene mutation (Paz-Filho et al, 2008). The current leptin therapy drug in use is metreleptin, brand name Myalept, for generalized lipodystrophy and congenital leptin deficiency (Paz-Filho, 2016). At this time, Maylept has only been FDA-approved for congenital or acquired generalized lipodystrophy (Paz-Filho, 2016).

As previously mentioned, administration of leptin through an injection directly into the hippocampus has been shown to have an antidepressant-like and anxiolytic-like effect in rats and mice as well as increase cognitive functions and in *ob/ob* mice, obesity and high corticosterone have been reduced after leptin injections were received (Collin et al, 2000; Lu et al, 2006; Harvey et al, 2005; Yamada et al, 2011; Guo et al, 2012; Sharma et al, 2010; Liu et al, 2010; Hwa et al, 1996; Mistry et al, 1997). Therefore, leptin presents as a very promising therapeutic agent for obesity, depression, anxiety, learning and memory.

Despite this evidence, leptin as a treatment presents a complicated problem. In obesity, leptin resistance develops such that administering leptin has no effect on weight loss (Chan et al, 2003). The benefits of leptin as a neuroprotective and synaptic plasticity-enhancing agent have been shown, however leptin could have dire effects on cancer cells due to its antiapoptotic effects (Oomura et al, 2006; Farr et al, 2006; Tang, 2008). Leptin is also similar in structure to cytokines and would have the potential to increase inflammation if administered chronically (Heshka and Jones, 2001). For example, a study has monitored inflammation markers such as CRP, TNFalpha and cortisol and no

difference was found in these particular measures from saline controls (Greco et al, 2010). However, there are many other pro-inflammatory cytokines that were not measured and leptin administration could impact levels of those cytokines which could lead to inflammation (Greco et al, 2010). There are also leptin receptors in the periphery and so leptin administration could result in side effects mediated through receptors there (Tartaglia et al, 1995; Lee et al, 1996; Fei et al, 1997; Ghilardi and Skoda, 1997; Hoggard et al 1997; Bjorbaek and Kahn, 2004).

The peptide nature of leptin also poses a problem for systemic injection since it is quickly and easily degraded. However, altering its transport in the blood can grant it a longer life and greater ability to interact with its receptors. A Pluronic block copolymer has been designed for this very purpose, Pluronic P85 (Price et al, 2010). The Pluronic P85 was combined with leptin and this resulted in the ability to pass through the BBB as well as a more than doubled half-life. To show the efficacy of the Pluronic P85 with leptin, evidence of a greater decrease in feeding in mice with the Pluronic leptin combination compared to vehicle was seen (Price et al, 2010). This indicates that the leptin did in fact reach the brain to decrease feeding behaviors. Although the effects of Pluronic leptin on learning and memory was not tested, it is feasible to suggest that Pluronic leptin would be able to enhance learning and memory in the hippocampus and have the other synaptic plasticity effects seen with direct leptin administration. Additionally, the actions of Pluronic leptin had a faster onset than leptin alone and continued to reach the brain and have effects at low and high doses, unlike leptin without Pluronic P85 and so could potentially be a useful therapeutic delivery system (Price et al, 2010).

CHAPTER 3

CIRCUITRY: AN INTRODUCTION TO NEURONAL ACTIVATION

As mentioned above, leptin administration to the hippocampus has been shown to enhance LTP (Shanley et al, 2001; Wayner et al, 2004), increase morphology changes and neurogenesis (O'Malley et al, 2007; Garza et al, 2008), and facilitate spatial learning and memory (Harvey, 2007; Farr et al, 2006; Li et al, 2002; Oomura et al, 2006). Models of leptin deficiency and resistance have further supported the importance of leptin in synaptic plasticity by showing deficits in electrophysiological, morphological and behavioral tests that are improved after leptin restoration (Winocur et al, 2005; Li et al, 2002; Grillo et al, 2011; Yamada et al, 2011). The effects of leptin when applied directly into the hippocampus have clear effects on synaptic plasticity; however, indirect pathways may be utilized by leptin that result in synaptic plasticity changes in the hippocampus. Due to the nature of the release of leptin from the peripheral adipocytes and the localization of leptin receptors throughout the brain, there is evidence that would support leptin mediating synaptic plasticity in the hippocampus through multi-synaptic circuitry.

3.1 NEURONAL ACTIVATION

In order to propose indirect circuits, the general effects of leptin on membrane potential and neurotransmitter systems in various brain regions must be analyzed. Leptin activates its leptin receptors on a neuron, resulting in signal cascades and release of neurotransmitter that can result in depolarization, hyperpolarization or no change in the membrane potential of the following neuron (Goforth et al, 2014; Spanswick et al, 1997; Cowley et al, 2001; van den Top et al, 2004; Lee et al, 2013; Powis et al, 1998; Dhillon et al, 2006). This results in a decrease, increase or no change in the following neuron's likelihood to fire an action potential and release neurotransmitter. Depending on what type of neurotransmitters a neuron containing leptin receptors releases, leptin binding can result in the release of GABA or glutamate, inhibitory and excitatory neurotransmitters, respectively (Vong et al, 2011; Goforth et al, 2014; Tong et al, 2007). Other neurotransmitters may also be released and result in neuromodulatory effects or membrane potential differences; however, GABA and glutamate are the most ubiquitous neurotransmitters used in the brain. Excitatory postsynaptic potentials (EPSPs) occur when the presynaptic neuron undergoes an action potential, releases neurotransmitter, and the resulting change in membrane potential of the postsynaptic neuron becomes more positive, towards zero from -70 mV. EPSPs can summate temporally or spatially to result in an action potential in the postsynaptic neuron, beginning the process all over again in the downstream neuron. Conversely, an inhibitory postsynaptic potential (IPSP) results in the postsynaptic neuron's membrane potential becoming more negative, hyperpolarized, and the neuron is less likely to fire. EPSPs and IPSPs summate continuously in neurons and result in a go or no-go firing response. Networks of neurons are able to be controlled

in such a way because neurons project axons to many dendrites of many different neurons, as many as 10,000 presynaptic neurons can project to a single neuron.

Calcium influx into the terminal is crucial for neurotransmitter release due to it being directly involved in neurotransmitter release from synaptic vesicles at the terminal of the axon boutons. Synaptic vesicles filled with neurotransmitter are located at the terminal ends of the axon boutons and vesicles undergo exocytosis after a series of events occurs. Calcium acts on synapsin, a protein involved in binding vesicles in the reserve pool and after a complex series of events, the vesicles are then free to dock to the plasma membrane and undergo exocytosis to release neurotransmitter into the synaptic cleft. Several proteins are involved with vesicle docking, including SNAP-25, increased by leptin application (Ahima et al, 1999). Once neurotransmitter is released into the synaptic cleft, it can act on the postsynaptic neuron and then be degraded or taken up by various enzymes or the presynaptic neuron or surrounding glia.

Leptin can bind to its receptors and activate signaling cascades in many areas of the brain, including the hippocampus, nuclei of the hypothalamus: VMH, DMH, lateral hypothalamus, ventral premammillary nucleus (PMv), arcuate, the ventral tegmental area (VTA), raphe nuclei, amygdala, and the brainstem (Elmquist et al, 1998; Morash et al, 1999; Ur et al, 2002; Hay-Schmidt et al, 2001; Elias et al, 2000; Figlewicz et al, 2003; Fulton et al, 2006; Grill et al, 2002; Hommel et al, 2006; Leinninger et al, 2009; Leshan et al, 2009; Mercer et al, 1996; Munzberg, 2008; Scott et al, 2009). In order to determine whether leptin binds to and activates receptors in brain regions that express the ObRb, several techniques are used to determine whether neurons are activated by leptin.

Neuronal activation can be assessed using electrophysiology, immunoassay for Fos, assessing neurotransmitter release, and behavioral changes.

The *c-Fos* gene is transcribed quickly after a stimulus occurs, first observed after nicotinic acetylcholine receptors were activated and *c-fos* gene transcription and subsequent Fos expression occurred (Greenberg et al, 1986). *c-Fos* expression has also been shown to occur after seizures and other stimuli and therefore Fos expression, measured with an immunoassay, is now used to indicate activated neurons (Morgan et al, 1987; Flavell and Greenberg, 2008). Peripheral leptin administration results in *c-Fos* expression in the hypothalamus, indicating activation of the neurons there (Woods et al, 1996; Elmquist et al, 1997).

Neurotransmitter release, for example of glutamate, is another way to assess whether neurons have been activated. Once neurons are sufficiently depolarized and calcium has entered the axon terminals, vesicles containing neurotransmitter undergo exocytosis and release transmitter into the synaptic cleft. Microdialysis is a technique that utilizes the principles of dialysis, movement of molecules and water through a semipermeable membrane, to withdraw neurotransmitter into a microdialysis probe and then analyze and qualify with high performance liquid chromatography (HPLC) (Parent et al, 2001). The probe is filled with solution that contains the same concentrations as fluid in the brain so that small particles such as neurotransmitters can diffuse into the probe (Parent et al, 2001). Glutamate release can be measured with microdialysis and indicates neuronal activation because a neuron must be depolarized, or activated, to release its transmitter. Leptin acting on receptors in the VMH results in excitation of glutamatergic neurons there with subsequent glutamate release on the downstream

neurons (Canteras et al, 1994; Wang et al, 2015). This glutamate release can affect many other brain areas further downstream, as the VMH projects to many other regions such as POMC neurons in the arcuate, and the hippocampus (Canteras et al, 1994). A subdivision of POMC neurons in the arcuate nucleus that leptin activates have been shown to release acetylcholine in addition to the POMC and CART peptides (Meister et al, 2006). Another study has shown about a third of POMC neurons express the GABA synthesis enzyme, glutamic acid decarboxylase (Hentges et al, 2004). These data show how many neurotransmitter types and systems that may be influenced by leptin.

Behavioral changes are another way to assess when neurons have been activated by observing how behaviors change over time after some stimulus. As previously stated, leptin has been shown to affect learning, memory and cognition in several behavioral tests. Leptin injections improve performance in the Morris Water Maze, T-maze footshock and passive avoidance tests (Oomura et al, 2006; Rasi et al, 2006; Garza et al, 2008; Farr et al, 2006). In leptin deficient or resistant animals, leptin restoration increases performance in several tests (Winocur et al, 2005; Li et al, 2002; Grillo et al, 2011; Yamada et al, 2011). Leptin also decreases depressive-like and anxiety-like behaviors in the forced swim test and also aids in stress management as seen in the chronic social defeat test (Lu et al, 2006; Liu et al, 2010; Sharma et al, 2010). These behavioral tests allow for changes at the neuronal level as a result of leptin to be seen on a macro scale.

3.2 EXCITATION & INHIBITION BY LEPTIN

As previously stated, the ObRb is paired with the JAK/STAT signaling pathway to affect gene transcription, a process that can take days (Ghilardi et al, 1996). However,

the ObRb also utilizes PI3K and MAPK signaling and studies show that leptin can bind to receptors and immediately alter firing patterns using these pathways (Bjorbaek and Kahn, 2004; Glaum et al, 1996; Powis et al, 1998; Cowley et al, 2001; Schwartz and Moran, 2002). It is by rapid signaling with the PI3K and MAPK pathways that leptin binding is able to immediately alter membrane potential and hyperpolarize or depolarize neurons.

Leptin can have an activation effect or inhibition effect using the same receptor, the ObRb, and different signaling pathways such as PI3K or MAPK. In addition to these levels of specificity, leptin can also activate a calcium-activated potassium channel involved in inhibition in the hippocampus (Spanswick et al, 1997; Shanley et al, 2002). Additional studies have shown that PI3K signaling is involved in the calcium-activated potassium channel's inhibition of hippocampal neurons, as the channel activation by leptin was blocked by PI3K antagonist wortmannin (Shanley et al, 2002). Calcium-activated potassium channel activation is dependent on calcium concentration and it has been proposed that leptin activates these channels as a result of the ability of leptin to increase intracellular calcium concentration (Oomura et al, 2006). Shanley and colleagues have proposed that NMDA receptor current is enhanced by leptin; however at rest, leptin inhibits hippocampal neurons through the calcium-activated potassium channels (Shanley et al, 2002).

Which brain areas and neuronal populations are excited or inhibited by leptin and which neurotransmitters are released in response to leptin binding is a current area of research. Studying activation and inhibition using Cre/lox technology has allowed much greater understanding of which neuronal populations that respond to leptin in the

hypothalamus are activated or inhibited by leptin binding and what neurotransmitter systems are involved. Vong and colleagues generated Vgat-ires-Cre and Vglut2-ires-Cre mice to express Cre recombinase in GABAergic or glutamatergic neurons, respectively (Vong et al, 2011). Crossing these mice with lox-green fluorescent protein (GFP) reporter mice allows for detection of GFP using immunohistochemistry and so the ability to determine which neurons are GABAergic or glutamatergic.

Studying colocalization of leptin receptor activity, using pSTAT3, and GFP expression in Vgat-ires-Cre mice showed which areas of the hypothalamus that contained GABAergic neurons with leptin receptors. Colocalization was found in the arcuate, DMH and lateral hypothalamus. Similar experiments were completed in Vglut2-ires-Cre mice and it was determined that the following areas had glutamatergic neurons with leptin receptors: a small part of the arcuate, VMH and PMv (Vong et al, 2011). These data show that leptin not only activates GABAergic neurons but also glutamatergic neurons in the hypothalamus that can project and release their respective neurotransmitters to many other brain regions, including the hippocampus (Canteras et al, 1994).

Vgat-ires-Cre and Vglut2-ires-Cre mice were also crossed with Lepr flox/flox mice to target leptin receptors from only GABAergic or glutamatergic neurons. This resulted in the mice that lacked leptin receptors on GABAergic neurons gaining a significant amount of weight. This led to an understanding that leptin predominantly utilizes GABAergic neurons for its regulation of body weight and energy expenditure (Vong et al, 2011). While it was determined in this set of experiments that leptin uses GABAergic inhibition for its food intake and energy balance effects, the effect of leptin on the glutamatergic population in the hypothalamus was not studied (Vong et al, 2011).

Another study knocked down glutamatergic neurons containing leptin receptors in the hypothalamus and saw that mice had lower body temperatures and energy expenditures compared to controls, as measured by oxygen consumption (Xu et al, 2013). It was determined that these glutamatergic neurons are important in thermoregulation and energy expenditure, however, no measures of synaptic plasticity were analyzed in the hippocampus. It is possible then that leptin binding to leptin receptors on glutamatergic neurons in the hypothalamus project to the hippocampus and utilize glutamate to affect synaptic plasticity.

To propose where a circuit influenced by leptin can begin, brain regions that express leptin receptors must be determined and then what effect leptin has once it activates those receptors, then subsequent projections and neurotransmitters. Leptin receptors have been found in the following hypothalamic regions: VMH, DMH, LH, and PMv. In addition, leptin receptors have been found in the hippocampus, VTA, the raphe nuclei, brainstem, parabrachial nucleus, periaqueductal gray, dorsal vagal complex (Hakansson et al, 1998; Elmquist et al, 1998; Burguera et al, 2000; Haung et al, 1996; Mercer et al, 1996; Leinninger et al, 2009; Scott et al, 2009; Grill et al, 2002; Elias et al, 2000; Figlewicz et al, 2003; Fulton et al, 2006; Hommel et al, 2006; Leshan et al, 2009; Munzberg, 2008;). These areas pose potential starting points for circuitry involving the hippocampus and synaptic plasticity there.

3.3 LEPTIN & OREXIN: APPETITIVE PEPTIDES WITH INDIRECT CIRCUITRY

The indirect routes that leptin may use to facilitate synaptic plasticity in the hippocampus are mirrored by the peptides orexin A and orexin B. Orexin A and orexin B are appetitive peptides that arise from the same *Ox* gene, cleaved to yield two peptides (Akbari et al, 2007). The orexin-1 receptor has a greater binding affinity for orexin A and the orexin-2 receptor has approximately the same affinity for both orexins (Sakurai et al, 1998). The orexins are produced by neurons located exclusively in the lateral hypothalamus and perifornical areas that stimulate feeding, wakefulness and are involved in sleep (Nambu et al, 1999; Peyron et al, 1998; Sakurai et al, 1998; de Lecea et al, 1998; O'Leary, 2014). Orexin is similar to leptin in that it may also utilize whole-brain circuitry, strengthening the argument that appetite and energy balance-related peptides are able to manipulate synaptic plasticity using brain-wide networks.

Orexin receptors are widespread throughout the brain, including the hippocampus, septum, arcuate nucleus, lateral hypothalamus, paraventricular nucleus of the hypothalamus, dorsal raphe nucleus and locus coeruleus (Cluderay et al, 2002; Wayner et al, 2004; Backberg et al, 2002; Akbari et al, 2006; Marcus et al, 2001). Orexigenic neurons from the lateral hypothalamus have been found to project to many areas of the hypothalamus, locus coeruleus, dorsal raphe, as well as the hippocampus (Date et al, 1999; Peyron et al, 1998). Interestingly, orexin A has been shown to be involved in hippocampal LTP, learning and memory, mirroring the roles of leptin in the hippocampus. For example, orexin A has also been shown to improve memory processing in the T-maze footshock avoidance and enhance LTP in the perforant pathway of the dentate gyrus (Akbari et al, 2006; Wayner et al, 2004; Jaeger et al, 2002). Orexin

administration to the dentate gyrus enhanced LTP and leptin also enhanced LTP dose-dependently, inhibiting LTP at the smallest and largest doses (Wayner et al, 2004). These data are consistent with another study showing that leptin facilitates and enhances LTP dose-dependently with an inverse U dose response curve (Wayner et al, 2004; Oomura et al, 2006). The orexin-1 receptor selective antagonist SB-334867 administered in the CA1 region of the hippocampus with MWM resulted in decreased time spent in the area where the platform had been and showed impaired acquisition (Akbari et al, 2006). These results show that the output of signaling with the orexin-1 receptor in the hippocampus is remarkably similar to that of leptin in terms of behavioral, learning and memory. This evidence that orexin affects synaptic plasticity in the hippocampus similarly to the way that leptin does is further evidence that orexin and leptin both work by direct and indirect circuitry.

Leptin and orexin appear to act in opposition in several brain areas, including the hypothalamus. Leptin has opposite effects on two different neuronal populations in the arcuate nucleus that project to the lateral hypothalamus, seen by Fos expression and tract-tracing (Elias et al, 1999). Leptin inhibits via hyperpolarizing AgRP/NPY neurons in the lateral hypothalamic area and medial arcuate nucleus while exciting neurons via depolarizing POMC/CART neurons in the medial and lateral arcuate (Elias et al, 1999; Cowley et al, 2001; Takahashi and Cone, 2005; van den Top et al, 2004). Orexin has an opposite effect, it can depolarize both AgRP/NPY and POMC/CART neurons (van den Top et al, 2004; Guan et al, 2001). The role of orexin in feeding behaviors act in opposition to leptin, to stimulate food intake and decrease energy expenditure (de Lecea

et al, 1998; Sakurai et al, 1998; Edwards et al, 1999). It would appear then that leptin and orexin have related, but opposite effects in the lateral hypothalamus.

One study looked at the effect of leptin and orexin administration in tissue slices of the arcuate nucleus in the hypothalamus and extracellular recordings showed that some of the same cells that were activated by orexin were also inhibited by leptin (Rauch et al, 2000). Orexin A excited around 85% of the neurons in the arcuate nucleus whereas leptin only elicited any change in about 24% of the neurons in the arcuate nucleus (Rauch et al, 2000). This is interesting to connect to another study's findings that the leptin receptors in the arcuate nucleus are primarily GABAergic with a small amount being glutamatergic (Vong et al, 2011). Interestingly, about half of the neurons were inhibited and half were excited and the longer leptin was present in the arcuate nucleus, the greater the inhibition (Rauch et al, 2000). These results show that leptin inhibited and excited a small amount of neurons in the arcuate and orexin only excited a much larger percentage, showing again an opposition in the same area and electrophysiological effects.

Interestingly, leptin has been shown to inhibit orexin neurons in the lateral hypothalamus, as seen in bath application of leptin in hypothalamic slices (Yamanaka et al, 2003). Leptin application causes hyperpolarization of orexin neurons and decreases the firing rate in a dose-dependent manner, higher levels of inhibition with higher doses of leptin (Yamanaka et al, 2003). It was also found that administering leptin to *ob/ob* mice increased *orexin* mRNA expression, while leptin given to wild-type mice resulted in suppression of *orexin* mRNA (Yamanaka et al, 2003). The difference is proposed as being related to the elevated glucose levels in *ob/ob* mice, that orexin neurons are inhibited in hyperglycemia, as pair-feeding *ob/ob* mice to wild-type mice, lowering food

intake, lowered blood glucose to normal levels and increased *orexin* mRNA levels (Yamanaka et al, 2003). The mechanism behind the ability of leptin to inhibit orexin neurons has been elucidated. Lateral hypothalamic neurons that express both leptin receptors and neurotensin are activated and subsequently hyperpolarize orexin neurons. This is further supported by an experiment showing that the removal of the leptin receptor from these neurons removes the hyperpolarizing effect on orexin neurons (Goforth et al, 2014). Interestingly, blocking GABA transmission did not remove the hyperpolarization effect of leptin and so it was determined that leptin application decreases EPSCs onto orexin neurons by lowering the amount of glutamate released onto orexin neurons and opening an ATP-sensitive potassium channel on orexin neurons (Goforth et al, 2014). These data show that leptin inhibits orexin neurons not with GABA or by leptin receptors located on orexin neurons but indirectly, through a potassium channel and by decreasing the frequency of EPSCs onto orexin neurons (Leinninger et al, 2009; Louis et al, 2010; Laque et al, 2013; Goforth et al, 2014).

Electrical stimulation of the lateral hypothalamus, locus coeruleus and other areas that contain both orexin and leptin receptors have been shown to affect synaptic plasticity in the hippocampus (Akbari et al, 2006; Frey et al, 2001; Walling et al, 2004; Wayner et al, 1997). The locus coeruleus produces norepinephrine, and norepinephrine in the dentate gyrus stimulates LTP. In this regard, orexin was directly injected into the locus coeruleus which resulted in norepinephrine release into the dentate gyrus which in turn enhanced EPSPs in the dentate gyrus (Walling et al, 2004). Similar results were seen when stimulating the basolateral amygdala in that transient LTP was seen 30 minutes after stimulating the basolateral amygdala with high frequency pulses (Frey et al, 2001).

Whole stimulation of the lateral hypothalamus results in blocking LTP in the dentate gyrus (Wayner et al, 1997). Leptin has been shown to act directly on its receptors in the lateral hypothalamus and these leptin-expressive neurons that colocalize with leptin receptors are GABAergic (Vong et al, 2011). This could be interpreted in a variety of ways, including that leptin inhibits inhibition of LTP in the dentate gyrus by activating GABAergic neurons in the lateral hypothalamus.

These stimulation studies are evidence that indirect pathways could be utilized by orexin and leptin and these pathways have already demonstrated that synaptic plasticity is affected through electrical stimulation. While the above studies are limited to only studying a few brain regions, it can be expected that synaptic plasticity would be affected if other brain regions were stimulated. This provides a strong argument that indirect pathways of leptin to influence synaptic plasticity are very likely used.

The mechanisms through which leptin and orexin act in opposition in many brain regions such as the arcuate nucleus of the hypothalamus while also having very similar actions on synaptic plasticity in the hippocampus could be an example of two different multi-synaptic sets of circuitry (Rauch et al, 2000; Akbari et al, 2006; Wayner et al, 2004; Jaeger et al, 2002). Evidence that stimulation of brain regions that contain both orexin and leptin receptors influences synaptic plasticity in the hippocampus provides further support that these appetitive peptides operate using several networks, starting in one brain region and ending in the hippocampus.

CHAPTER 4

INDIRECT PATHWAYS FOR LEPTIN TO AFFECT SYNAPTIC PLASTICITY

4.1 LEPTIN RECEPTORS IN THE VENTRAL TEGMENTAL AREA

Leptin receptors and leptin receptor mRNA have been found in the ventral tegmental area (VTA), an area composed of dopaminergic neurons involved reward, emotion, and other behaviors (Leshan et al, 2010; Figlewicz et al, 2003; Fulton et al, 2000; Fulton et al, 2006; Leinninger et al, 2009; Hommel et al, 2006; Ranaldi, 2014). The VTA, substantia nigra and the projections from both are part of the mesolimbic dopamine system. These dopaminergic neurons project to many regions including the striatum, amygdala, nucleus accumbens and hippocampus to the subiculum and areas CA1 and CA3 (Bjorklund and Dunnett, 2007a; Leshan et al, 2010; Lammel et al, 2008; Gasbarri et al, 1991).

Leptin has been shown to directly activate dopaminergic neurons (Hommel et al, 2006; Fulton et al, 2006; Leinninger et al, 2009). The activation of leptin receptors directly on VTA neurons was assessed by looking at whether STAT3 was phosphorylated after leptin administration (Ghilardi et al, 1996; Hommel et al, 2006). Leptin was injected directly into the VTA and significant pSTAT3 was seen on tyrosine hydroxylase-containing neurons, neurons that contain the enzyme to synthesize dopamine (Hommel et al, 2006). Interestingly, direct injection of leptin into the VTA resulted in less food

intake, a behavioral effect similar to that of leptin injections into the arcuate nucleus of the hypothalamus (Elmquist et al, 1998; Huang et al, 1996).

The electrophysiological effects of leptin on dopaminergic VTA neurons was assessed both *in vivo* and *in vitro* with single-cell recordings and in brain slices, respectively. *In vivo*, leptin decreased the frequency of dopaminergic neuron firing and *in vitro,* bath application of leptin resulted in a hyperpolarization of the membrane (Hommel et al, 2006). These data show that leptin directly acts in the VTA to influence behavior as well as firing rate of the neurons there. These experiments did not include an assessment of any synaptic plasticity effects as a result of direct application of leptin in the VTA but due to the measured effects on feeding behavior and firing rate and the projections from the VTA to the hippocampus, it is possible synaptic plasticity in the hippocampus could be altered by this pathway.

Dopaminergic neurons project to the subiculum, to the cells of the dentate gyrus and pyramidal neurons in areas CA1 and CA3 and these dopaminergic projections have been shown to affect synaptic plasticity in the form of LTP (Hortnagl et al, 1991; Verney et al, 1985; Gasbarri et al, 1991; Jay, 2003). Antagonism of dopamine receptors in area CA1 results in failure to maintain LTP and agonists of dopamine receptors in area CA1 increase the slope of EPSPs (Huang and Kandel, 1995; Frey et al, 1990). LTP in response to dopamine has been assessed in the dentate gyrus but to mixed results; one found no effect, another found LTP to not be induced with agonist but lengthened the time LTP was present, some found LTP was induced with agonist (Swanson-Park et al, 1999; Yanagihashi and Ishikawa, 1992; Kusuki et al, 1997). It appears, then, that dopamine acting on dopamine receptors in area CA1 increases the slope EPSPs and blocking

dopamine receptors negatively effects LTP maintenance whereas in the dentate gyrus, LTP was induced or lengthened depending on the study. The effects of leptin on membrane potential and firing rate in the VTA which projects directly to the hippocampus in combination with the effects of dopamine on hippocampal LTP provides a potential indirect route through which leptin may affect hippocampal synaptic plasticity (Leinninger et al, 2009; Hortnagl et al, 1991; Verney et al, 1985; Gasbarri et al, 1991; Miler and Bacon, 1989).

It should be noted that this information conflicts with the observation that *ob/ob* mice have lower levels of tyrosine hydroxylase mRNA compared to wild-type, the lack of leptin in circulation would seem to result in more tyrosine hydroxylase and potentially more dopamine release due to the lack of inhibition in the VTA reported in the study (Hommel et al, 2006; Fulton et al, 2006; Leinninger et al, 2009). The electrophysiological studies also conflict with another study that shows that systemic injections as well as hypothalamic injections of leptin result in increased amphetamine-induced dopamine release in the nucleus accumbens (Perry et al, 2010). These studies differ on the manner of leptin application, indirect vs direct in the VTA resulting in more and less release of dopamine, respectively. These conflicting results support leptin using different circuitry to exert different effects, depending on the location of leptin binding.

To further support that leptin could increase dopamine levels in the mesolimbic system by some pathways, activation of leptin-receptor containing neurons that project from the lateral hypothalamus to the VTA have been shown to increase the enzyme involved in dopamine production in the VTA (Leinninger et al, 2009; Leshan et al, 2010; Hommel et al, 2006). Direct injection of leptin into the lateral hypothalamus resulted in

increased pSTAT3 in the lateral hypothalamus and increased tyrosine hydroxylase levels in the VTA in both *ob/ob* and wild-type mice (Leinninger et al, 2009). Leptin injected peripherally resulted in dopamine release into the nucleus accumbens and enhanced dopamine transporter and tyrosine hydroxylase activity in the nucleus accumbens (Perry et al, 2010). This shows that leptin receptor-containing neurons from the lateral hypothalamus not only are being activated by leptin but project to the VTA to increase the levels of dopamine-synthesizing enzyme to potentially alter dopamine levels in the mesolimbic system. The differences in direct electrophysiological effects of leptin in the VTA and systemic or hypothalamic injection of leptin on dopamine release has sparked controversy in the field and perhaps networks of inhibitory and excitatory effects of leptin in the VTA or projecting to the VTA play a role in altering synaptic plasticity in the hippocampus.

Leptin receptor-containing neurons from the VTA also project to the central amygdala (Leshan et al, 2010). The amygdala, specifically the basal nuclei, indirectly project to the hippocampus through the prelimbic prefrontal cortex (McDonald et al, 1996; Vertes, 2004). Leptin administered to *ob/ob* mice resulted in an increase in phosphorylated CREB, an indicator of activity, showing that leptin increased activity in the neurons in the central amygdala (Leshan et al, 2010). The lateral hypothalamus also projects to the amygdala, providing an additional indirect route to the hippocampus (Hahn and Swanson, 2015; Goto et al, 2005). This could result in several pathways activated by leptin, beginning in the central amygdala to the basal nuclei and ultimately to the hippocampus.

4.2 LEPTIN RECEPTORS IN THE LOCUS COERULEUS

The locus coeruleus is the main center in the brain for production of norepinephrine, classically involved in attention and memory and there is significant norepinephrine input to the hippocampus, specifically to the adrenergic receptors in the dentate gyrus (Loughlin et al, 1986; Booze et al, 1993). The locus coeruleus also expresses leptin receptors, as seen by immunohistorychemistry studies double-labeling leptin receptors and tyrosine hydroxylase and the dorsal locus coeruleus projects to the hippocampus (Hay-Schmidt et al, 2001; Elmquist et al, 1998; Loughlin et al, 1986). The direct effects of leptin in the locus coeruleus have not been explored; however there are projections from leptin-receptor containing neurons from the lateral hypothalamus to the locus coeruleus (Laque et al, 2015). This could be a route by which leptin could regulate the locus coeruleus that in turn goes on to project to the hippocampus (Loughlin et al, 1986).

Norepinephrine has been implicated in LTP in the perforant pathway of the dentate gyrus (Walling et al, 2003). Norepinephrine-induced LTP can be blocked by the same NMDA receptor antagonist that blocks LTP in the dentate gyrus, D-(-)-2-amino-5-phosphonovaleric acid, demonstrating norepinephrine's importance to LTP in the dentate gyrus (Dahl et al, 1989). Depleting norepinephrine with 6-OHDA resulted in a decrease of frequency of LTP in the dentate gyrus only, no change in frequency seen in LTP in area CA1 (Stanton and Sarvey, 1985). Other studies have found that stimulating the locus coeruleus results in inhibition or excitation of the dentate gyrus and others have found norepinephrine activating its receptors in the dentate gyrus to induce LTD (Segal and Bloom, 1976; Dahl and Winson, 1985; Hansen and Manahan-Vaughan, 2015).

One group looked at the effects of leptin on catecholamine release when leptin is injected i.c.v. into the VMH, arcuate, paraventricular and dorsomedial hypothalamic nuclei (Satoh et al, 1999). It was found that leptin injected into the VMH resulted in increases in plasma norepinephrine and epinephrine proportional to the dose of leptin injected. The other areas of the hypothalamus did not see such increases (Satoh et al, 1999). This provides evidence that leptin has influence over norepinephrine release mediated through the VMH of the hypothalamus that could bind to adrenergic receptors in the hippocampus and induce LTP or LTD (Loughlin et al, 1986; Booze et al, 1993).

Lesions of the locus coeruleus have resulted in less preference for sucrose and less food intake that reversed 5 days later, showing that the locus coeruleus is similarly involved in the modulation of feeding behaviors and therefore could also be involved in leptin regulating synaptic plasticity (Ammar et al, 2001). Treatment with an alpha 2 receptor agonist, clonidine, resulted in less food intake in both *ob/ob* and wild-type mice; however, another study used an alpha 2 receptor antagonist that reduced food intake while clonidine increased carbohydrate and total food intake (Currie and Wilson, 1991; Currie and Wilson, 1992). Although these results are inconsistent with each other, they show that leptin may have some control over the locus coeruleus in feeding behaviors and therefore also control over locus coeruleus' projections to the hippocampus.

4.3 LEPTIN RECEPTORS IN THE RAPHE NUCLEUS

The raphe nucleus is the primary site of synthesis for serotonin, a neurotransmitter involved in feeding, sleep and depressive disorders (Lam and Heisler, 2007; Lucki, 1998; Pinder and Wieringa et al, 1993; Jouvet et al, 1967). A high level of immunoreactivity of

the leptin receptor has been located in the raphe and double label *in situ* hybridization has shown that leptin receptor-containing neurons that express the serotonin transporter mRNA are present in the caudal linear, dorsal and medial raphe although there is some controversy over this (Hay-Schmidt et al, 2001; Finn et al, 2001; Lam et al, 2011). The dorsal and medial areas of the raphe, have been shown to project directly to the hippocampus, hypothalamus and amygdala, providing an indirect route to the hippocampus through which leptin can act (Vertes et al, 1999; Azmitia and Segal, 1978; Sawchenko et al, 1983; Willoughby and Blessing, 1987; Donovan and Tecott, 2013; Petrov et al, 1992).

In support of this hypothesis, digoxigenin-labelled leptin was injected into the lateral cerebral ventricle and confocal microscopy was used to identify that labelled leptin had accumulated in serotonergic neurons of the dorsal raphe (Fernandez-Galaz et al, 2002). To further support that leptin acts in the raphe nucleus, an injection of leptin into the brainstem has been shown to activate leptin receptors on serotonergic neurons in the dorsal and medial raphe nucleus of wild-type mice, confirmed by immunohistochemistry for pSTAT3 (Yadav et al, 2009). Leptin injected i.c.v. was found to significantly correlate with higher levels of brain serotonin after three hours (Calapai et al, 1999). It was also found that, with *in situ* hybridization, *ob/ob* mice have less serotonin transporter mRNA compared to controls, implying a relationship between leptin and serotonin (Collin et al, 2000). Additionally, deletion of leptin receptors on serotonergic neurons in the brainstem resulted in decreased energy expenditure and the development of obesity, further supporting this relationship (Yadav et al, 2009). These data demonstrate that leptin activates serotonergic neurons in the raphe and that in *ob/ob* mice, serotonin

transporter mRNA expression is decreased, indicating an interaction between leptin and serotonergic neurons.

Leptin has been shown to have electrophysiological effects in wild-type mice, however this data also comes with some controversy. Leptin bath application has been shown to decrease the frequency of action potentials of serotonergic neurons in brain slices with the application of phenylephrine, an alpha-1 adrenergic receptor agonist (Yadav et al, 2009). Another study reported no change with bath application of leptin on brain slices of wild-type mice, however phenylephrine was not added to the bath (Lam et al, 2011; Yadav et al, 2009). The former paper has added that serotonergic neurons have lost norepinephrine input in slices and therefore are dormant without the adrenergic receptor agonist, a more physiologically relevant observation (Yadav et al, 2009). These electrophysiological data show that leptin directly effects serotonergic neurons that go on to project to the hippocampus (Yadav et al, 2009; Vertes et al, 1999; Azmitia and Segal, 1978; Sawchenko et al, 1983; Willoughby and Blessing, 1987; Donovan and Tecott, 2013; Petrov et al, 1992).

Serotonin acting on the different serotonin receptor sub-types have been found to alter both glutamate and GABA release in different brain regions. In the cortex and septum, serotonin binding to the 5-HT2 receptor increases glutamate release while in the cerebellum, decreases glutamate release (Aghajanian and Marek, 1997; Hasuo et al, 2002; Torres-Escalante et al, 2004; Maura et al, 1991). In contrast, serotonin binding to the 5-HT3 receptor increases GABA in area CA1 while antagonism of the 5-HT7 receptor in area CA3 alters GABA-mediated activity (Gill et al, 2002; Katsurabayashi et

al, 2003). It would seem that serotonergic effects depend on both brain region and serotonin receptor subtype.

Serotonergic neurons innervate the dentate gyrus and have been shown to increase neurogenesis there (Malberg et al, 2000). Additionally, inhibiting serotonin synthesis decreased the rate of neurogenesis in the dentate gyrus, showing the importance of serotonin in neurogenesis (Brezun and Daszuta, 1999; Djavadian, 2004). Interestingly, depletion of serotonin using 5,7-DHT or PCPA in hippocampal slices did not affect frequency of LTP in either the dentate gyrus or area CA1 and only amplitude was non-significantly decreased with PCPA (Stanton and Sarvey, 1985). In hippocampal slices, the application of exogenous serotonin or serotonin receptor agonists have resulted in inhibition (Mlinar et al, 2003; Jahnsen, 1980, Pugliese et al, 1998). In contrast, application of 3,4-methylenedioxymethamphetamine (MDMA) to trigger endogenous release of serotonin resulted in facilitation of greater LTP in areas CA3 and CA1 (Rozas et al, 2011). The difference between these two results seems to depend on exogenous vs endogenous serotonin and that using MDMA to result in the release of serotonin from axons in areas CA3 and CA1 results in greater LTP (Mlinar et al, 2015). Endogenous serotonin, then, appears to enhance both LTP and neurogenesis and so the raphe nucleus is a candidate for a potential indirect pathway for leptin to influence synaptic plasticity in the hippocampus.

4.4 LEPTIN RECEPTORS IN THE LATERAL HYPOTHALAMUS

The hippocampus is increasingly considered as being involved in feeding behaviors and energy regulation, in addition to its roles in learning and memory

(Davidson et al, 2005, Davidson et al, 2007). Reciprocal connections from area CA1 of the hippocampus to various nuclei in the hypothalamus have been identified. Area CA1 projects directly to the VMH, lateral hypothalamus and the midline thalamic nuclei (Cenquizca and Swanson, 2007). It has been suggested that these projections may be involved in motivated behaviors, such as feeding (Cenquizca and Swanson, 2007). Additionally, the VMH, lateral hypothalamus and midline thalamic nuclei send projections to the hippocampus (Lima et al, 2013; Canteras et al, 1994). To further support the connection of the hippocampus to the hypothalamus, it has been shown that lesioning projections from the hippocampus to the PVN resulted in a significant increase in CRH mRNA expression in the PVN (Herman et al, 1992). This demonstrates a direct influence of the hippocampus on an area of the hypothalamus that has further downstream effects on the hypothalamic-pituitary-adrenal axis (HPA) and release of cortisol from the adrenals. The lateral hypothalamus has both GABAergic and glutamatergic neuronal populations and have been shown to be involved in feeding (Jennings et al, 2013; Wu et al, 2015). These data show that the hippocampus is directly connected to the hypothalamus and due to leptin receptors being located in both the hypothalamus and hippocampus, there could be bidirectional effects of leptin in these brain regions (Morash et al, 1999; Ur et al, 2002).

Studies have recently elucidated a pathway from the ventral hippocampus to the dorsal perifornical lateral hypothalamus activated by ghrelin, an appetitive hormone from the stomach (Tschop et al, 2000; Cowley et al, 2003; Hsu et al, 2015). Ghrelin is proposed as being involved in meal anticipation, as deletion of its receptor blocks anticipation of food (Blum et al, 2009). The ventral hippocampus has leptin and ghrelin

receptors and the ventral hippocampus projects directly to the lateral hypothalamus, discovered using tract tracing and immunohistochemistry approaches (Hsu et al, 2015). Additionally, in the same set of experiments, it was found that blocking ghrelin receptors in the ventral hippocampus resulted in decreased food intake when rats were only allowed to feed for one 4 hour-window each day after meal entrainment. These data show how an appetite and energy-related hormone utilizes a direct route from the hippocampus to the lateral hypothalamus for meal anticipation and food intake, evidence that leptin could be using the same pathway to effect synaptic plasticity changes in the hippocampus.

Leptin is similarly involved in feeding behaviors and so could be acting through this pathway as well. Several groups have explored the role of leptin in the ventral hippocampus. Leptin also has a role in a type of learning and memory, food-related spatial learning and memory that is mediated by the ventral hippocampus specifically (de Hoz et al, 2003; Bannerman et al, 2004; Kanoski et al, 2011). The dorsal hippocampus is correlated with spatial memory and the ventral hippocampus is correlated with emotional and motivational memory (de Hoz et al, 2003; Bannerman et al, 2004; Fanselow and Dong 2010; Kanoski et al, 2011). Leptin injected directly into the ventral hippocampus resulted in decreased food intake, similar to leptin injections into the hypothalamus (Kanoski et al, 2011). This would indicate a link between the leptin, ventral hippocampus and hypothalamus.

Conditioned Place Preference (CPP) is a hippocampal-dependent test designed to link a rewarding event, such as food, with the location in the environment where the rewarding event took place (Ito et al, 2006). When leptin was again injected directly into

the ventral hippocampus 3 hours prior to the CPP test, the food-related conditioned place preference was inhibited, compared to vehicle controls. Leptin administered directly into the dorsal hippocampus did not see such an inhibition. So then, memory consolidation for the location of the food was only impaired when leptin was injected into the ventral hippocampus and not the dorsal hippocampus. The activation of the leptin receptors in the ventral hippocampus in this experiment are thought to block the recall of memories related to food by cues in the environment (Kanoski et al, 2011).

Leptin in the ventral hippocampus not only has an impact on food-related behaviors but also memory recall. And while synaptic plasticity measures such as LTP or non-food related behavioral studies were not conducted in this experiment, it is likely synaptic plasticity changes compared to controls would have been seen. The authors suggest that the hypothalamus is not the only brain region involved in regulation by leptin of food intake and energy balance which can translate to regulation of synaptic plasticity by leptin, dispersed throughout the brain and connected through many synapses. Leptin very likely utilizes a network, not just one brain region, for its effects on both food intake and metabolism but also synaptic plasticity and this idea has been suggested by several other studies in relation to the effects of leptin on energy expenditure and food intake (Leinninger and Myers, 2008; Myers et al, 2009).

4.5 LEPTIN RECEPTORS IN THE PARAVENTRICULAR HYPOTHALAMUS

The paraventricular nucleus of the hypothalamus (PVN) is involved in controlling pituitary, feeding and autonomic functions (van den Pol and Trombley, 1993; Buijs et al, 2003; Coote, 1995; Ferguson et al, 2008). There are many populations of neurons within

the PVN, including neurons that contain corticotropin-releasing factor (CRF), thyrotropin releasing hormone (TRH) and oxytocin (Perello and Raingo, 2013; Legradi et al, 1997; Herman et al, 1989). Neurons that contain glutamate or GABA have also been identified in the PVN, glutamatergic and GABAergic neurons were identified with immunocytochemistry for rapid excitatory and inhibitory transmission, respectively, in the PVN (van den Pol and Trombley, 1993; Decavel and van den Pol, 1990).

Leptin application results in changes in membrane potential in hypothalamic brain slices that included the PVN (Powis et al, 1998). Whole-cell recordings were completed and bath application of leptin resulted in depolarized the majority of neurons and this effect was dose-dependent (Powis et al, 1998). To further investigate the effect of leptin on current, it was found that bath application of leptin increased the amplitude of inward current (Powis et al, 1998). These results are supported by a study that found Fos expression in the PVN after leptin was administered peripherally (Elmquist et al, 1997). These data show that leptin directly activates neurons in the PVN, mediated through leptin receptors there (Elmquist et al, 1998; Elmquist et al, 1997). A study has found projections from the PVN to area CA2 of the hippocampus, using retrograde tracers (Cui et al, 2013). In addition, the PVN projects to many areas that in turn project to the hippocampus, including: the DMH, VMH, lateral septum, bed nucleus of the stria terminalis, amygdala, pituitary gland, spinal cord and several others (Moga et al, 1995; Swanson and Kuypers, 1980). The electrophysiological data in combination with direct and indirect projections of the PVN to the hippocampus result in several potential pathways that leptin can activate to induce synaptic plasticity changes in the hippocampus.

Leptin receptors are found in the PVN on a variety of different neurotransmitter-containing neurons, including neurons that contain TRH (Legradi et al, 1997). Release of TRH from the PVN regulates the release of thyroid stimulating hormone (TSH) from the pituitary gland which then stimulates the thyroid to synthesize and release T3 and T4, the thyroid hormones (Flier et al, 2000). TRH release is reduced during fasting, when leptin levels are low. Leptin activates TRH-containing neurons and can therefore regulate the hypothalamic-pituitary-thyroid axis (HPT) axis. Thyroid hormone levels increase with leptin treatment after fasting rats and leptin application results in TRH release in hypothalamic cell cultures (Ahima et al, 1996; Legradi et al, 1997; Blake et al, 1991; Kim et al, 2000; Nillni et al, 1999). Another study has shown that leptin administration brought T4 and T3 levels back to normal after animals were fasted, supporting the ability of leptin to affect change in the HPT (Legradi et al, 1997). ProTRH gene expression in the PVN is reduced during fasting and i.p. leptin has been shown to increase proTRH back to normal levels, showing that leptin can regulate expression of thyroid hormone mRNA. These data show that leptin can regulate thyroid hormones and the HPT axis and activate TRH-containing neurons in the PVN.

As mentioned above, leptin stimulates TRH release which acts through the HPT axis to result in the synthesis and release of T3 and T4, which have been shown to act in the hippocampus. T3 and T4 have been shown to decrease GABAergic currents in hippocampus cell cultures, thereby establishing another circuit that leptin could use to reduce inhibitory tone in the hippocampus to potentially pave the way for excitation and synaptic plasticity (Puia and Losi, 2011). To further establish the importance of thyroid hormones in the hippocampus, it has been shown in gestation day 6-old prenatal rats and

30 day-old postnatal rats that hypothyroidism can impair synaptic plasticity at area CA1 of the hippocampus (Sui and Gilbert, 2003). The effect of neonatal hypothyroidism can extend to adulthood, as a study has found that LTP and synaptic transmission were impaired in adult rats (Gilbert and Paczkowski, 2003). This evidence of dysregulated thyroid hormones can show how leptin influence is important in LTP.

In addition to the ability of leptin to regulate the HPT axis through neurons in the PVN, it has also been shown that leptin can regulate oxytocin-containing neurons of the PVN. Levels of pSTAT3 were increased in oxytocin neurons, indicating that leptin had activated these neurons (Perello and Raingo, 2013). Leptin also regulates oxytocin mRNA expression in that fasted rats had significantly lower levels of oxytocin mRNA in the PVN compared to non-fasted controls. Interestingly, DIO rats showed activation of some of the oxytocin-containing neurons by i.c.v. leptin, as evidenced by pSTAT3 (Perello and Raingo, 2013). This is an interesting result due to the decreased leptin signaling normally found in DIO animals and although not all of the oxytocin neurons by were activated by i.c.v. leptin, some were. This could mean that not all leptin signaling is inhibited in a leptin-resistant state, at least in some populations of the hypothalamus. (Perello and Raingo, 2015).

The ability of leptin to influence oxytocin release may be another indirect route that is used by leptin to facilitate synaptic plasticity in the hippocampus. Oxytocin receptors have been found on the hippocampus and animals given intranasal oxytocin performed better in the MWM (Lee et al, 2015). Neurogenesis has been shown to be affected by oxytocin, oxytocin was applied to a neural stem cell line and there was an increase in cell proliferation of progenitor cells (Musaelyan et al, 2011). These data show

that leptin can influence oxytocin neurons that can subsequently influence synaptic plasticity in the hippocampus, specifically enhancing learning and memory performance and stimulating neurogenesis in adults (Lee et al, 2015; Musaelyn et al, 2011).

Leptin receptors on TRH and oxytocin neurons show that leptin binding to its receptors in the PVN can regulate systems like the HPA and HPT and the release of many neurotransmitters which could in turn regulate synaptic plasticity in the hippocampus (Legradi et al, 1997; Perello and Raingo, 2013). Perhaps in addition to direct communication with the hippocampus using GABA and glutamate, leptin in the PVN also activates the release of other neurotransmitters that modulate synaptic plasticity. Additional hormones and systems that leptin can regulate in the PVN include corticotropin-releasing factor (CRF) and subsequent ACTH release from the pituitary, vasopressin, neurotensin and many others (Herman et al, 1989).

4.6 LEPTIN RECEPTORS IN THE DORSOMEDIAL HYPOTHALAMUS

The dorsomedial nucleus of the hypothalamus (DMH), associated with food and water intake, has been shown to express leptin receptors (Bellinger and Bernardis, 2002; Schwartz et al, 1996; Elmquist et al, 1998). Immunohistochemistry and retrograde transport of cholera toxin b were used to determine which areas of the hypothalamus were excited by leptin administration, revealing double-labeling of Fos and the cholera toxin in the DMH (Elmquist et al, 1997). This indicates that leptin activated neurons in the DMH.

The neurotransmitter phenotype of DMH neurons is relatively unstudied, however a microarray study found that DMH neurons that contained NPY expressed glutamic acid

decarboxylase, suggesting that these neurons are GABAergic (Draper et al, 2010). Another study utilized transgenic mice to express GFP in choline acetyltransferase (ChAT) positive neurons, and found that cholinergic neurons are present in the DMH and are involved in energy homeostasis (Groessl et al, 2013). Additionally, tract tracing has revealed that the DMH projects to the PVN and releases both GABA and glutamate, additionally the DMH expresses both glutamate and GABA receptors (Myers et al, 2014; Silva et al, 2015; Boudaba et al, 1996; Ulrich-Lai et al, 2011).

The projections of the DMH were investigated using an adeno-associated virus that encoded GFP in combination with Cre recombinase so that only neurons in the DMH that also expressed leptin receptors would express Cre (Gautron et al, 2010). GFP expression was present in axonal projections of neurons with cell bodies in the DMH and the following brain regions contained terminals from the DMH: heavy projections to the arcuate, PVN, preoptic area, bed nucleus of the stria terminalis, supraoptic nucleus, lateral septum, parts of the thalamus, periacqueductal gray and precoeruleus areas. Less strong innervation was seen to the lateral hypothalamus, mammillary nuclei and dorsal raphe nucleus (Gautron et al, 2010).

The exact significance of these projections has yet to be determined, but it does establish several potential novel routes that leptin activates to have downstream effects in the hippocampus (Elmquist et al, 1997). The projections to the dorsal raphe nucleus and precoeruleus areas would add another layer of serotonergic and noradrenergic control, respectively, to the leptin receptors present in the raphe nucleus and locus coeruleus (Hay-Schmidt et al, 2001; Finn et al, 2000; Elmquist et al, 1998; Loughlin et al, 1986). The projections to the PVN and lateral hypothalamus may also add to the complexity of

leptin acting through various hypothalamic regions to control synaptic plasticity in the hippocampus (Gautron et al, 2010).

4.7 LEPTIN RECEPTORS IN THE VENTROMEDIAL HYPOTHALAMUS

The ventromedial hypothalamic nucleus is involved in feeding and energy homeostasis, as lesions of this area result in obesity (Choi et al, 2013; Balagura 1970; Becker et al, 1974; Berthoud et al, 1979; King 2006). One study found a lack of obesity with lesioning of the VMH, however the findings of this study were unable to be replicated and have since been called into question (Gold et al, 1973). The VMH is also involved in fear responses, seen with an increase in Fos expression in the dorsomedial area of the VMH after mice were allowed to interact with a predator rat (Silva et al, 2013). Interestingly, the same *c-Fos* activation was not seen during foot-shock (Silva et al, 2013). Because of this, the authors suggest a role for the VMH in social fear, which other studies support (Perez-Gomez et al, 2015). Thermoregulation, sexual activity and sympathetic control have also been found to involve the VMH, showing that the VMH plays a role in a variety of functions (Resch et al, 2011; Musatov et al, 2006).

The VMH releases glutamate, as seen by mRNA expression of VGLUT2, and projects to many regions, including the locus coeruleus, POMC neurons in the arcuate, and the hippocampus (Canteras et al, 1994; Ovesjo et al, 2001; Ziegler et al, 2002; Tong et al, 2007). The ventromedial nucleus projects directly to the entorhinal area of the hippocampus and it has been suggested that additional indirect routes to the hippocampus from the VMH nucleus are possible, mediated through the midline thalamic nuclei, amygdala, piriform area, endopiriform nucleus, and insula (Canteras et al, 1994). Other

regions that the VMH project to include the medial and lateral hypothalamus, bed nuclei of the stria terminalis and the periaqueductal gray (Canteras et al, 1994; Saper et al, 1976).

Leptin plays a role in the VMH, specifically with glutamatergic steroidogenic factor-1 (SF1) -containing neurons and are involved in regulation of glucose levels (Dhillon et al, 2006; Tong et al, 2007; Vong et al, 2011). Electrophysiological whole-cell recordings revealed that leptin depolarizes and increases the frequency of action potentials in SF1 neurons (Dhillon et al, 2006). Other roles for SF1 neurons include body weight regulation, as evidenced by a mouse model lacking leptin receptors exclusively on SF1 neurons resulted in an increase in body weight as compared to controls (Dhillon et al, 2006). These data show that the lack of leptin binding disrupted the role of leptin to decrease feeding. Although no synaptic plasticity changes were measured, the connections of the glutamatergic VMH neurons to the hippocampus could also have been adversely affected by the knockout of leptin receptors.

In addition to leptin receptors on SF1 neurons, there are leptin receptors expressed widely in the VMH and these receptors are actively used, according to a study that looked at Fos expression and *socs-3* mRNA (Bjorbaek et al, 1999; Elmquist et al, 1997). Leptin activates glutamatergic neurons in the VMH in addition to the glutamatergic SF1 neuronal sub-population (Tong et al, 2007). These data are important in establishing a potential pathway starting in the VMH that leads to glutamate release in the hippocampus to stimulate NMDA-receptor mediated synaptic plasticity. In addition, the large number of brain regions that the VMH projects to could be a starting point for a network of excitatory influence on synaptic plasticity.

These studies are significant in establishing an excitatory, glutamatergic indirect route that leptin can use to access the hippocampus. Leptin is excitatory on SF1 neurons in the VMH and other neurons in the VMH are glutamatergic as well, increasing the likelihood that leptin can result in glutamate release in the hippocampus to interact with NMDA and AMPA receptors. The implications of this excitatory input on synaptic plasticity in the hippocampus as a result of leptin binding in the VMH has not yet been explored but it is likely that this glutamatergic input upregulates synaptic plasticity.

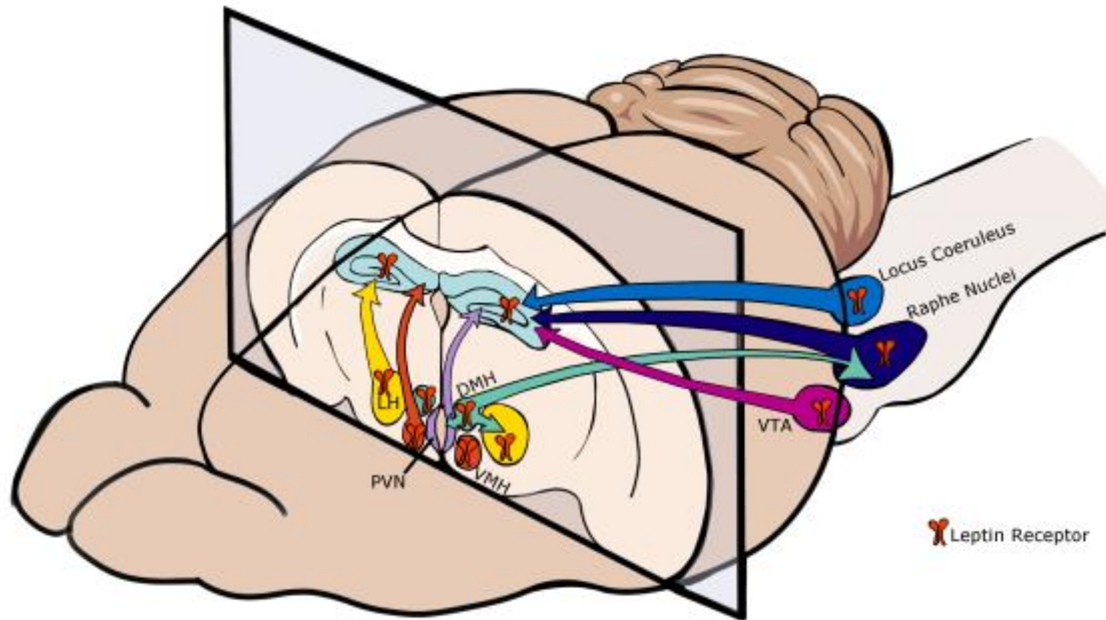

Figure 4.1. Potential indirect pathways that leptin uses to influence synaptic plasticity in the hippocampus.

Table 4.1. Indirect pathways to the hippocampus with potential neurotransmitters released

Brain Region	Projects Directly to Hippocampus	Potential Neurotransmitters Released
Lateral Hypothalamus	Yes	Glutamate, GABA
DMH	No	Glutamate, GABA
PVN	Yes	Glutamate, GABA, Oxytocin, TRH
VMH	Yes	Glutamate
VTA	Yes	Dopamine
Raphe Nuclei	Yes	Serotonin
Locus Coeruleus	Yes	Norepinephrine

CHAPTER 5

CONCLUSIONS & FUTURE DIRECTIONS

Leptin has direct effects on synaptic plasticity in the hippocampus that clearly demonstrate the ability of leptin to enhance LTP, convert STP to LTP, traffic glutamatergic receptors to the membrane, increase calcium influx into the presynaptic neuron, increase dendritic spine density and increase cell proliferation (Oomura et al, 2006; Wayner et al, 2004; Shanley et al, 2001; Garza et al, 2008; Dhar et al, 2014; O'Malley et al, 2007; Moult et al, 2010). In addition, leptin administration improves performance in several hippocampal-dependent learning and memory behavioral tests (Oomura et al, 2006; Rasi et al, 2006; Garza et al, 2008; Farr et al, 2006). The lack of leptin or impaired leptin signaling results in deficits in each of these measures of synaptic plasticity (Li et al, 2002; Dhar et al, 2014; Pinto et al, 2004; Ahima et al, 1999; Li et al, 2002; Winocur et al, 2005; Grillo et al, 2011). Therefore, leptin has significant direct effects on synaptic plasticity, including learning and memory in the hippocampus.

Leptin is primarily peripherally synthesized, crosses the BBB and the long form of the leptin receptor mRNA and protein is expressed widely throughout the brain (Frederich et al, 1995; Banks et al, 1996; Hileman et al, 2002; Banks et al, 2006; Hakansson et al, 1998; Elmquist et al, 1998; Burguera et al, 2000; Huang et al, 1996; Hay-Schmidt et al, 2001; Ur et al, 2002; Figlewicz et al, 2003; Fulton et al, 2006; Leinninger et al, 2009; Hommel et al, 2006). Through several studies with immunocytochemistry to study the activation of these receptors by leptin with labeling

for pSTAT3, it has been confirmed that leptin utilizes these receptors and signals with them using the JAK/STAT signaling pathway, other studies have shown leptin receptors paired with the MAPK or PI3K pathways (Vong et al, 2011; Hommel et al, 2006; Leininger et al, 2009; Yadav et al, 2009; Perello and Raingo, 2013; Bates et al, 2003; Zhao et al, 2002b). Leptin binding to the ObRb has also been shown to alter membrane potential to either depolarize or hyperpolarize neurons (Cowley et al, 2001; van den Top et al, 2004; Guan et al, 2001; Powis et al, 1998; Dhillon et al, 2006; Goforth et al, 2014). Therefore, leptin acts in many regions of the brain and can result in excitation or inhibition of neurons.

The hypothalamus in particular has a high concentration of leptin receptor mRNA and a variety of effects as a result of leptin binding to its receptor (Elmquist et al, 1998; Cowley et al, 2001; van den Top et al, 2004; Goforth et al, 2014). The hypothalamus also projects widely, many nuclei sending reciprocal projections to each other as well as regions such as the hippocampus (Canteras et al, 1994; Cenquizca and Swanson, 2007; Herman et al, 1992; Morash et al, 1999; Ur et al, 2002; Tschop et al, 2000; Hsu et al, 2015). It is proposed, then, that the hypothalamus acts as a central nexus that regulates many of the effects of leptin, be it food intake, energy expenditure or LTP in the hippocampus. The large number of regions that the VMH projects to in combination with its high level of glutamate release could be a jumping point to a broad network of a excitatory influence by leptin on synaptic plasticity (Canteras et al, 1994; Ovesjo et al, 2001; Ziegler et al, 2002; Tong et al, 2007). Leptin activating or inhibiting neurons in the lateral hypothalamus that project to the amygdala, VTA, nucleus accumbens, locus coeruleus, raphe, and even other nuclei in the hypothalamus supports the idea of a

network that begins in the hypothalamus and projects widely, ultimately influencing synaptic plasticity through many circuits (Hahn and Swanson, 2015; Goto et al, 2005; Bjorklund and Dunnett, 2007a; Leshan et al, 2010; Lammel et al, 2008). The hypothalamus could be acting as a main point that not only diverges out to the rest of the brain but also a point that other areas converge to for synthesis of information. The hypothalamus could use multi-synaptic circuitry, by projecting to an area that subsequently projects to another to then finally to the hippocampus, creating a multiplying effect of the number of pathways that lead back to the hippocampus. The circuits proposed, with beginning points in the VTA, locus coeruleus, raphe nucleus, VMH, DMH, PVN and lateral hypothalamus provide strong evidence that leptin uses a combination of direct and indirect circuits for its effects.

It has been proposed that leptin activates or inhibits different circuits for the various effects of leptin on feeding, energy balance, reproduction, temperature regulation, and neuroendocrine control (Zhang et al, 1994; Flak and Myers, 2015). There are many brain regions involved in just control of feeding, including the arcuate, lateral hypothalamus, DMH, VMH, raphe nucleus, locus coeruleus and many others. Therefore, it is likely that leptin also utilizes its hypothalamic circuitry that projects to the hippocampus indirectly to enhance synaptic plasticity (Canteras et al, 1994; Gautron et al, 2010).

The disperse nature throughout the brain of the feeding circuits alone lends credit to the idea that the regulation of synaptic plasticity is also disperse and that many interconnected brain regions are involved. For example, there are two groups of neurons in the arcuate nucleus that contain leptin receptors and are controlled by leptin in opposite

ways. The medial arcuate nucleus contains AgRP and neuropeptide Y (NPY) containing neurons while the lateral arcuate nucleus contains POMC neurons (Flak and Myers, 2015). Leptin excites POMC neurons that release a-MSH, POMC, CART, GABA, acetylcholine while leptin inhibits medial arcuate nucleus AgRP neurons that can release AgRP, NPY and GABA (Bouret et al, 2004; Vong et al, 2011; Cowley et al, 2001; Kalra et al, 1999; Cone, 1999; Zigman and Elmquist, 2003). Both POMC and AgRP neurons project to three other food-related nuclei: PVN, DMH and lateral hypothalamus as well as many other brain regions (Bouret et al, 2004; Elias et al, 1999). This is only part of the circuitry involved in the regulation of feeding and demonstrates how far-reaching and complex the circuit can be. Synaptic plasticity circuits are likely just as complex and utilize several brain regions, neuron types and neurotransmitter systems. This idea is further supported by a study where deletion of STAT3 signaling in only the arcuate nucleus resulted in a small impact on body weight and feeding, illustrating how not just one region with leptin receptors signaling with the JAK/STAT pathway is responsible for body weight control (Gong et al, 2008; Buettner et al, 2006; Xu et al, 2007). This could apply to how leptin regulates several circuits and that the hippocampus is not the only brain region for leptin to act in to affect synaptic plasticity.

The nature of brain is such that different brain regions are interconnected and many of the regions that express leptin receptors project to the hippocampus (Hortnagl et al, 1991; Verney et al, 1985; Loughlin et al, 1986; Vertes et al, 1999; Azmitia and Segal, 1978; Sawchenko et al, 1983; Willoughby and Blessing, 1987; Donovan and Tecott, 2013; Petrov et al, 1992). Other studies have found both leptin mRNA or leptin receptors and Fos expression in other areas of the hypothalamus, including the VMH and PVN,

showing that leptin activates each of these areas (Elmquist et al, 1998; Schwartz et al, 1996; Elmquist et al, 1997; Mercer et al, 1996). Determination of activation in the hypothalamus by leptin using a Fos and cholera toxin showed that leptin activated neurons in the DMH. However, the absence of Fos expression in other brain areas may indicate that leptin may have been inhibitory (Elmquist et al, 1997). Hyperpolarization in the hypothalamus after leptin application, seen with electrophysiology techniques, supports this hypothesis (Elmquist et al, 1997; Spanswick et al, 1997; Yamanaka et al, 2003; Goforth et al, 2014). This shows that leptin both excites neurons and inhibits neurons even within just one brain area, the hypothalamus. Leptin exciting one area of the brain could cause downstream excitation in the hippocampus, enhancing synaptic plasticity indirectly. Conversely, leptin could inhibit an area of the brain, resulting in downstream lack of excitation in the hippocampus, either downregulating or reversing synaptic plasticity changes. This is an important function of leptin, the ability to both depolarize or hyperpolarize, depending on the location of the receptor in a brain region. This could allow for the balance of enhancements in learning and memory, a homeostatic type of regulation that is prevalent throughout the brain and body. In addition, the inhibitory effects of leptin allow for fine-tuning of excitation, to allow the execution of synaptic plasticity changes only when necessary or past a certain threshold. The manner in which leptin facilitates synaptic plasticity is in line with its characteristic of being neuromodulatory, not only directly altering synaptic plasticity but tweaking other areas for a combined effect.

Interestingly, several studies have reported that GABAergic neurotransmission is what predominately regulates feeding behaviors in the hypothalamus while also reporting

glutamatergic transmission that does not play much of a role except in preventing hypoglycemia (Vong et al, 2011; Tong et al, 2007). The effects of this glutamatergic transmission on synaptic plasticity in the hippocampus have not yet been studied, due to the nature of the studies focusing on feeding and energy expenditure. However, it certainly possible that the glutamate transmission in the hypothalamus influences the hippocampus. Some studies have even shown the relationship between the hippocampus and hypothalamus and how the hippocampus is involved with food-related memory and even regulation of the HPA axis by projections to the PVN (Bannerman et al, 2004; Kanoski et al, 2011, Herman et al, 1992; Myers et al, 2014; Herman et al, 1989). Glutamatergic transmission in the hypothalamus could be involved in the regulation of the HPA axis and food hippocampal-dependent learning and memory. This demonstrates the interconnected nature and complexity of the HPA axis and food intake regulation networks and how it is likely that hippocampal synaptic plasticity regulation is similarly complex and widespread.

Leptin has been shown to result in an inhibitory effect in some brain areas while having an excitatory effect in others, perhaps to balance its effects on feeding or synaptic plasticity so that neither is constantly being up- or down-regulated. Additionally, leptin could begin a network of excitatory pathways that begin in the hypothalamus, resulting in regions that were previously inhibited by leptin by direct action in these regions subsequently being depolarized through glutamatergic neurotransmission to induce synaptic plasticity in the hippocampus. To support this, other studies have suggested the idea that leptin acts through neural networks which contain leptin receptors (Fadel et al, 2013; Leinninger and Myers, 2008; Myers et al, 2009; Scott et al, 2009). The distributed

model proposed by these studies for the feeding actions of leptin is applicable to the synaptic plasticity actions of leptin. The excitatory pathways beginning in the VMH, DMH, LH or PVN could be tempered by inhibition of excitatory neurotransmission coming from the locus coeruleus, raphe or VTA. These networks would work in harmony and be controlled by leptin.

Future studies should take measures of hippocampal synaptic plasticity, for example using electrophysiology to measure LTP or conduct hippocampal learning and memory-dependent behavioral studies after directly injecting leptin into one of the potential areas that would begin a circuit. Confirmation of circuits could provide the knowledge of how normal physiology works to allow for greater understanding of pathology and the development of better treatment of diseases where leptin is dysregulated, such as diabetes, obesity, metabolic syndrome, cognitive disorders and Alzheimer's Disease.

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