An Examination of the Role of Brain-derived Neurotrophic Factor in Neural Circuits Controlling Homeostatic and Hedonic Food Intake

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Abstract

Food intake is a complex behavior coordinated, in part, by homeostatic mechanisms in the hypothalamus balancing nutritional and caloric requirements. Hedonic pleasure derived from indulging in palatable food is mediated by a midbrain circuit known as the mesolimbic dopamine pathway. Brain-derived neurotrophic factor (BDNF) and its receptor, TrkB, are critical components within these tightly regulated circuits controlling food intake. Evidence from rodent and human studies establish perturbed BDNF signaling as a biological risk factor contributing to obesity and its associated medical complications. However, the mechanisms underlying the satiety effects of BDNF and its role in the pathogenesis of obesity are not completely understood.

In this dissertation, I investigate a mechanism mediating the satiety effects of BDNF in the ventromedial hypothalamus (VMH), a region critical to maintaining energy homeostasis. I demonstrate that reduced cell surface expression of α2δ-1, a calcium channel subunit and thrombospondin receptor, contributes to the overeating and obesity triggered by BDNF deficiency ($BDNF^{2L/2LCk\text{-}cre}$) in mice. Accordingly, pharmacological inhibition of α2δ-1 in wild type VMH induces overeating and weight gain. Furthermore, viral-mediated rescue of α2δ-1 in the BDNF mutant VMH attenuated excessive feeding and obesity and significantly improved metabolic function. These findings demonstrate a previously unrecognized role of α2δ-1 in appetite control facilitated by BDNF.

I also present work investigating the regulatory effects of BDNF in the mesolimbic dopamine pathway and hedonic feeding control. I demonstrate that defective dopamine transmission underlies the pathological consumption of palatable food in $BDNF^{2L/2LCk\text{-}cre}$ mice. Indeed, the hyperphagic behavior exhibited by $BDNF^{2L/2LCk\text{-}cre}$ mice could be

overcome by administration of a selective dopamine agonist. I also show that selective *Bdnf* depletion in the ventral tegmental area, the origin of mesolimbic dopamine fibers, significantly increases palatable food consumption leading to obesity. These data indicate that BDNF signaling in the mesolimbic pathway is essential for hedonic feeding control. In summary, the work presented in this dissertation provide novel, mechanistic insight into the anorexigenic effects of BDNF in homeostatic and hedonic feeding circuits, introducing new pharmaceutical strategies for the global obesity epidemic.

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List of commonly used abbreviations

α2δ-1, alpha 2 delta-1

AgrP, agouti-related protein

Arc, arcuate nucleus of the hypothalamus

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BDNF, brain-derived neurotrophic factor

CART, cocaine- and amphetamine-regulated transcript

Ca_V, high voltage-gated calcium channel

CCK, cholecystokinin

CREB, calcium responsive element binding protein

CRH, corticotrophin-releasing hormone

DA, dopamine

DAT, dopamine transporter

D#R, dopamine receptor #

DMH, dorsomedial nucleus of the hypothalamus

DVC, dorsal vagal complex

GABA, γ-amino-butyric acid

GP, Gabapentin

HFF, high-fat diet

JNK, jun-N terminal kinase

LH, lateral nucleus of the hypothalamus

LTD, long-term depression

LTP, long-term potentiation

MAPK, mitogen-activated protein kinase

MCH, melanin-concentrating hormone

MC4R, melanocortin receptor 4

NAc, nucleus accumbens

NGF, nerve growth factor

NMDA, N-methyl-D-aspartic acid

NPY, neuropeptide Y

NT-3, neurotrophin 3

NT-4/5, neurotrophin 4/5

$p75^{NTR}$, pan-neurotrophin receptor 75

PFC, prefrontal cortex

PGB, pregabalin

PI-3K, phosphoinositide-3 kinase

PLC-γ1, phospholipase C γ1

POMC, pro-opiomelanocortin

PVN, paraventricular nucleus of the hypothalamus

SC, standard chow

Trk, tropomyosin-related kinase

TSP#, thrombospondin family member #

VMH, ventromedial nucleus of the hypothalamus

VTA, ventral tegmental area

Chapter 1:

Introduction

Overview of Neurotrophins

Neurotrophins are a family of structurally related growth factors serving diverse roles in the development, maturation, and survival of cells in the nervous system. Since the discovery of nerve growth factor (NGF) in the 1950's, the family has grown to include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Although principally found in mammals, reptiles, and amphibians, two additional neurotrophins closely related to NGF have been described exclusively in teleost fish: neurotrophin-6 (NT-6) in platyfish (Gotz & Schartl, 1994) and neurotrophin-7 (NT-7) in zebrafish and carp (Nilsson, Fainzilber, Falck, & Ibanez, 1998).

Current investigations of DNT1, a neurotrophin present in *Drosophila melanogaster*, further suggest that neurotrophin signaling is not a uniquely vertebrate innovation (Zhu et al., 2008). Nevertheless, the emergence of neurotrophins early in evolution and the selective pressure to conserve its presence throughout vertebrate phylogeny provides evidence for important neurotrophic roles in animal survival (Tettamanti et al., 2010). Indeed, loss of NGF, BDNF, or NT-3 genes is lethal in mouse knockouts models (Crowley et al., 1994; Donovan, Hahn, Tessarollo, & Hempstead, 1996; Ernfors, Lee, & Jaenisch, 1994). The high degree of neurotrophin sequence homology among vertebrates (the peptide sequences of which are essentially identical between mouse and human) moreover suggest that the essential functions for neurotrophins are similar among higher organisms (Kullander, Carlson, & Hallbook, 1997) and allow us to extrapolate key details about neurotrophin signaling in human diseases by using animal models.

Nerve growth factor, the first neurotrophin discovered, was initially described by Levi-Montalcini and colleagues as a substance capable of stimulating nerve fiber outgrowth in chick embryos, *in vitro* (Cohen, Levi-Montalcini, & Hamburger, 1954; Levi-Montalcini & Cohen, 1956). Subsequent investigations by Cohen and colleagues showed dramatic atrophy and destruction of the mammalian sympathetic nervous system in response to delivery of anti-NGF antibodies, indicating neurotrophin action was also required during development *in vivo* (Cohen & Levi-Montalcini, 1956; Levi-Montalcini & Cohen, 1956).

This evidence, together with the demonstrations that NGF alleviated normally occurring neuronal death in developing sensory and sympathetic neurons *in vitro* (Hamburger, Brunso-Bechtold, & Yip, 1981; Levi-Montalcini & Angeletti, 1963) and *in vivo*, formed the basis of the "Neurotrophin Hypothesis," championed by Victor Hamburger. Its central tenet states that the survival of developing neurons, which are produced in overabundance during neural development, depends on the successful competition for a limited supply of target-derived trophic factor(s) (Hamburger et al., 1981; Jones & Reichardt, 1990). Unsuccessful neurons, which do not make or maintain synaptic contact, experience pruning and cell death via apoptosis– the normal fate of up to 80% of all developing neurons. This is thought to serve as a mechanism for regulating the innervation density of target neuron populations (Jones & Reichardt, 1990).

Early studies revealed that not all target neuron populations responded to NGF, suggesting the potential for other neurotrophic factors (Lindsay, Barde, Davies, & Rohrer, 1985). Accordingly, a second neurotrophic factor, BDNF, was purified and cloned from pig brain and shown to support the survival and outgrowth of cultured sensory neurons in the chick embryo (Barde, Edgar, & Thoenen, 1982; Leibrock et al., 1989). *In vivo*, BDNF

significantly reduced the total number of degenerating neurons in Quail dorsal root ganglia (M. M. Hofer & Barde, 1988), further establishing BDNF's role as a neurotrophic factor. Soon after, NT-3 (Hohn, Leibrock, Bailey, & Barde, 1990; Jones & Reichardt, 1990; Maisonpierre, Belluscio, Squinto et al., 1990; Rosenthal et al., 1990) and NT-4/5 (Berkemeier et al., 1991; Hallbook, Ibanez, & Persson, 1991; Ip et al., 1992) were also identified as members of the neurotrophin family based on sequence homology and their ability to support the survival and growth of neurons *in vitro* and *in vivo*.

Altogether, NGF, BDNF, NT-3, and NT-4/5 define a family of structurally and functionally related protein dimers (McDonald et al., 1991; Robinson, Radziejewski, Stuart, & Jones, 1995). Within the most highly conserved region of this protein family, which are 50-55% homologous, 35 amino acids are invariant (Ip et al., 1992). Of these, six cysteine residues are key to the formation of disulfide bridges which together form a "cysteine knot" characteristic of all neurotrophins (Maisonpierre, Belluscio, Squinto et al., 1990). Despite their structural similarity, individual neurotrophins provide functional support to distinct yet overlapping populations of neurons in the central (CNS) and peripheral (PNS) nervous systems (A. M. Davies, Thoenen, & Barde, 1986; Lindsay et al., 1985). This evidence supports the notion that primary sensory neurons have a multiple growth factor requirement during development. In particular, BDNF acts as a common "central target-derived" (CNS) neurotrophic factor while peripheral classes (PNS) of sensory neurons depended on specific trophic factors including NGF, NT-3, and NT-4/5.

Individual neurotrophins can be further distinguished based on their patterns of temporal and spatial expression. NT-3, BDNF, and NGF transcripts all display a simultaneous, dramatic increase in expression between the eleventh and twelfth day of rat

embryogenesis (Maisonpierre, Belluscio, Friedman et al., 1990). NT-3 mRNA is by far the most abundant in early embryogenesis, whereas BDNF is expressed at the lowest levels. However, BDNF content in the brain increases dramatically during the post natal period, soon becoming the most abundant and highly distributed neurotrophic factor in the adult brain. In the adult hippocampus, a common site for the highest expression of all neurotrophic factors, BDNF is ~50 fold more abundant than NGF (M. Hofer, Pagliusi, Hohn, Leibrock, & Barde, 1990; Maisonpierre, Belluscio, Friedman et al., 1990). BDNF also displays a widespread distribution pattern with the highest levels of mRNA and protein in neurons of the hippocampus, amygdala, cerebral cortex, and hypothalamus, where its actions have been extensively studied (M. Hofer et al., 1990).

The Actions of BDNF

The actions of BDNF signaling have been the best characterized of the neurotrophin family. Initially identified as a growth factor supporting the survival of sensory neurons, BDNF is now recognized as a multifaceted trophic factor regulating the survival, differentiation, maturation, and synaptic plasticity of active neuronal circuits throughout life (Barde et al., 1982; Huang & Reichardt, 2001). Recent evidence from our lab, for example, has identified BDNF as a key regulator during the maturation, differentiation and dendrite development of adult-born neurons in the dentate gyrus of the hippocampus (Chan, Cordeira, Calderon, Iyer, & Rios, 2008). Moreover, BDNF, which is synthesized, stored and released by glutamate neurons, has been shown to mediate excitatory and inhibitory synaptic transmission (Lessmann & Heumann, 1998) by enhancing pre-synaptic glutamate release

(Takei et al., 1998) and by controlling post-synaptic NMDA, AMPA, and $GABA_A$ receptor activity at the cell membrane (Caldeira et al., 2007; Hewitt & Bains, 2006; Levine, Crozier, Black, & Plummer, 1998; Narisawa-Saito et al., 2002). Together, this identifies BDNF as a critical factor influencing long-term synaptic connectivity and function of the brain during development and in adulthood.

Indeed, alterations in BDNF signaling in the brain have been linked to numerous clinical disorders. A single nucleotide polymorphism (SNP) located in the 5' pro-region of *Bdnf* at nucleotide 196 (G/A) produces a valine to methionine amino acid substitution at codon 66 (Val66Met a.k.a. 196G/A). This SNP inhibits the activity-dependent trafficking and secretion of BDNF and has been linked to anxiety, depression, memory dysfunction, as well as abnormal feeding behavior and obesity (Z. Y. Chen et al., 2004; Z. Y. Chen et al., 2006; Gratacos et al., 2007). Investigating the mechanisms regulating BDNF expression, signaling, and how its dysfunction may contribute to the pathogenesis of these disorders is an important and exciting avenue of research.

Regulation of BDNF Expression

The mouse and rat *Bdnf* gene structure is highly complex and contains multiple regulatory elements. Located on the second chromosome, it consists of at least eight 5' untranslated exons (I-VIII) and a single protein coding 3' exon (IX). Transcription is initiated from multiple promoters upstream of these distinct exons to produce a heterogeneous population of BDNF mRNA. Additional elements within the exon/intron structure also add to this heterogeneity. For example, alternative splice donor sites within

exon II leads to three different exon II transcript variants (IIA, IIB, and IIC). *Bdnf* transcription can also be initiated in the intron immediately preceding the 3' exon resulting in IXA. There are also two alternative polyadenylation signals sites in the 3' exon. All told, there are at least 22 unique transcriptional units for *Bdnf*. Remarkably though, because each of the 5'exons is spliced to the common 3' protein coding exon, each transcript encodes an identical BDNF protein (Aid, Kazantseva, Piirsoo, Palm, & Timmusk, 2007).

The human *Bdnf* gene, located on chromosome 11, has 11 exons and nine functional promoters which can be processed to produce a diversity of 34 *Bdnf* transcripts, each encoding the same BDNF protein. Unlike the mouse or rat, human *Bdnf* can be bi-directionally processed to produce anti-sense BDNF transcripts capable of binding and regulating the translational potential of BDNF; thus adding another level of regulatory complexity (Pruunsild, Kazantseva, Aid, Palm, & Timmusk, 2007).

All of the 5' exons within the rodent *Bdnf* gene are controlled by distinct, activity-dependent and tissue-specific promoters which determine the spatial and temporal expression of BDNF (Timmusk et al., 1993; Timmusk, Belluardo, Persson, & Metsis, 1994). The *in vivo* relevance of this complexity isn't completely understood. However, BDNF splice variants show distinct expression profiles in the brain, spinal cord and non-neuronal tissue including the heart, lung, and liver and even kidney, muscle and testis. For example, transcripts I and IV are highly expressed in the ventromedial nucleus of the hypothalamus (VMH), whereas III is highest in the hippocampus and IV in the lung (Aid et al., 2007; Tran, Akana, Malkovska, Dallman, Parada, & Ingraham, 2006b).

Many factors have been reported to control the activity-dependent, tissue-specific, and sub-cellular expression of BDNF transcript. In response to neural activity, calcium influx rapidly phosphorylates and activates cAMP/Ca^{2+} response element binding protein (CREB), which activates *Bdnf* gene transcription by binding to a calcium response element in the *Bdnf* gene promoter III (Tao, Finkbeiner, Arnold, Shaywitz, & Greenberg, 1998). Moreover, an estrogen responsive element identified on the 5' end of the coding *Bdnf* exon (Pruunsild et al., 2007) induces transcriptional activation of *Bdnf* via estrogen receptor alpha transcription factor (Blurton-Jones, Kuan, & Tuszynski, 2004). Discrete, sub-cellular localization of BDNF mRNA is determined by alternative polyadenylation signals producing either short or long BDNF transcripts. Whereas short 3'UTR species localize to the soma, long 3'UTR BDNF mRNA is targeted to dendrites for local translation of BDNF protein involved in dendrite remodeling and hippocampal long-term potentiation (LTP) (An et al., 2008). These data offer exciting preliminary insight into the complex yet functionally relevant regulation of BDNF transcription.

Regulation of BDNF at the protein level is also functionally important. BDNF protein is predominantly produced by neurons, but is also expressed and released by astrocytes under the control of neuronal activity to a lesser extent (Zafra, Lindholm, Castren, Hartikka, & Thoenen, 1992). It is synthesized as a 32-kD N-glycosylated precursor molecule, pro-BDNF, which undergoes proteolytic cleavage to produce mature, homodimeric protein (Mowla et al., 2001). Pro-BDNF traffics from the ER to the *trans*-Golgi network where it is cleaved by furin or pro-convertases and sorted and packaged into vesicles for release (Lessmann, Gottmann, & Malcangio, 2003). Cleavage of precursor protein can also occur extracellularly and is mediated by plasmin (K. Gray & Ellis, 2008). Unlike other

neurotrophins which are constitutively secreted, BDNF is primarily packaged into secretory or large dense core vesicles for regulated, activity-dependent secretion in neurons. The Val66Met polymorphism in the pro-domain of BDNF impairs the intracellular trafficking and secretion of BDNF by preventing pro-domain interaction with sortilin, a chaperone protein important to directing BDNF for regulated secretion (Z. Y. Chen et al., 2004; Z. Y. Chen et al., 2005; Egan et al., 2003).

Following constitutive or regulated secretion from post-synaptic neurons, BDNF binds and activates its receptors, tropomyosin-related kinase receptor B (TrkB) and the P75 neurotrophin receptor ($P75^{NTR}$), at pre-synaptic terminals. Ligand-receptor complexes are subsequently internalized into signaling endosomes and retrogradely trafficked to the cell body, where they initiate local signal transduction cascades and gene transcription to mediate the long-term survival effects of neurotrophins (Lessmann et al., 2003). Retrograde transport of BDNF was confirmed electrophysiologicaly by Magby et al. in 2006 when they showed that tonic depolarization of a post-synaptic neuron evoked calcium-dependent release of BDNF, which bound pre-synaptic TrkB to elevate mEPSC frequency, a measure for pre-synaptic transmitter release (Magby, Bi, Chen, Lee, & Plummer, 2006). In addition to acting as a target-derived factor, BDNF can also undergo anterograde transport from the cell soma to axon terminals. This afferent supply of BDNF facilitates its neurotransmitter-like effects in post-synaptic targets (Altar et al., 1997).

It had long been thought (and recently debated) that only secreted, mature BDNF was biologically active. Work by Matsumoto et al. in 2008 showed that pro-BDNF was rapidly and intracellularly converted to mature BDNF, indicating that only mature BDNF was stored in vesicles and released by excitatory input (Matsumoto et al., 2008). However, other lines

of evidence show that pro-BDNF can also be secreted and then extracellularly cleaved by plasmin or matrix metalloproteinase to produce mature BDNF. Alternatively, pro-BDNF can also be secreted without subsequent cleavage and serve as a biologically active signaling molecule (K. Gray & Ellis, 2008; R. Lee, Kermani, Teng, & Hempstead, 2001; Mowla et al., 2001). Its activation of $P75^{NTR}$ antagonizes the actions of mature BDNF-TrkB complexes including cell survival (R. Lee et al., 2001; Teng et al., 2005). Whereas pro-BDNF is highly expressed during early postnatal stages, mature BDNF is most prominent in the adult brain (J. Yang et al., 2009). This is consistent with important roles for both pro-BDNF and BDNF in the maintenance or loss/pruning of selective target cell populations during early development and adulthood. The activation of $P75^{NTR}$ by pro-BDNF also facilitates hippocampal long-term depression (LTD) (Woo et al., 2005) arguing for a bidirectional role in synaptic plasticity; LTD by pro-BDNF and LTP by BDNF. Clearly, the cleavage of pro-BDNF is an important mechanism determining the biological activities of the mature protein.

BDNF Receptor Signaling

The diverse biological activities of BDNF, like other neurotrophins, are governed by interactions with their receptors tropomyosin-related kinase receptor (Trk) and the P75 neurotrophin receptor ($P75^{NTR}$) and activation of distinct intraceullular signaling cascades (Fig 1). Unlike the $P75^{NTR}$ receptor, which strongly binds all pro-neurotrophins, each class of Trk receptor (TrkA, TrkB, or TrkC) preferentially binds and is activated by different mature neurotrophins (Huang & Reichardt, 2003). NGF activates TrkA while BDNF and

NT-4/5 activate TrkB. NT-3 activates TrkC but can also minimally bind TrkA and TrkB (A. M. Davies, Minichiello, & Klein, 1995; Patapoutian & Reichardt, 2001).

Expression of Trk receptor transcripts is widespread in all neural tissue whereas expression in non-neuronal tissue is extremely limited (ex. skin, spleen, muscle) (Lomen-Hoerth & Shooter, 1995). Analysis of the developmental expression profiles for TrkA TrkB and TrkC are consistent with the patterns of their respective neurotrophins. While TrkA expression is more prominent in early CNS development, TrkB, similar to BDNF, is the most highly expressed Trk receptor in the brain (Valenzuela et al., 1993). The temporal expression profile for $P75^{NTR}$ is similar to that of TrkA. During developmental periods of naturally occurring cell death, $P75^{NTR}$ mRNA and protein levels are high (Buck, Martinez, Black, & Chao, 1987). They decrease into adulthood where steady state $P75^{NTR}$ expression is widespread in both neuronal and non-neuronal adult tissue (Lomen-Hoerth & Shooter, 1995).

TrkB Signaling

The crystal structure of the TrkB receptor and its ligand binding domain has been solved (Ultsch et al., 1999). Other members of the Trk family have similar properties. TrkB is a single-pass transmembrane molecule with an N-terminal extracellular domain and C-terminal cytoplasmic domain. The extracellular domain of TrkB contains an array of three leucine rich motifs flanked by two cysteine rich domains at the N-terminus. A pair of immunoglobin-like domains proximal to the membrane acts as major interfaces for neurotrophin binding (Ultsch et al., 1999). The intracellular domain of TrkB contains a

tyrosine kinase domain with ten conserved tyrosine residues critical to the transduction/initiation of intracellular signaling.

Truncated isoforms of TrkB lacking the tyrosine kinase domain have also been identified (Klein, Conway, Parada, & Barbacid, 1990). Both full-length and truncated receptor isoforms are co-expressed and located uniformly throughout neurons (Kryl et al., 1999) while glia express only truncated Trk (Valenzuela et al., 1993). The signaling and function of truncated TrkB isoforms aren't completely understood. While truncated TrkB may regulate full-length Trk signaling in neurons by sequestering ligand (Biffo, Offenhauser, Carter, & Barde, 1995) or dominant negative repression (Eide et al., 1996), they also have autonomous roles mediating dendrite outgrowth in select neuronal populations (Yacoubian & Lo, 2000) and intracellular calcium release in astrocytes (Rose et al., 2003). This is an important area for future research particularly since the expression of truncated TrkB, which begins postnatally, ultimately exceeds that of full-length TrkB in adulthood (Valenzuela et al., 1993).

In response to neurotrophin binding, full-length TrkB homodimerizes (Jing, Tapley, & Barbacid, 1992) and is *trans*-phosphorylated within the tyrosine kinase domain. Activated, phospho-tyrosines serve as specific docking sites for the intermediary signaling molecules phospholipase C-gamma (PLC-γ) and Src homology domain-containing protein (Shc). Subsequent initiation of PLC-γ, mitogen-activated protein kinase (MAPK) and phosphatydalinositol-3 kinase (PI3-K) signaling cascades promote a wide array of cellular functions leading to neuronal differentiation, survival, and synaptic plasticity in active neurons (Fig 1.1) (Patapoutian & Reichardt, 2001). The downstream consequences of TrkB

activation varies in a cell-type specific manner depending on which tyrosine becomes phosphorylated and the expression of the intermediary signaling molecules.

Tyrosine phosphorylation at Y515 on the cytoplasmic domain of TrkB recruits interaction with Shc. Shc, in turn, can activate the small GTP binding protein Ras through the intermediary molecule Grb-2 and the nucleotide exchange factor SOS (Basu, Warne, & Downward, 1994). Ras directs neuronal survival by directing neurotrophin-initiated signals into either of two signaling pathways, MEK/MAPK and PI-3K/Akt. GTP bound Ras activation of the Raf-Mek-MAPK kinase cascade can directly activate transcription factors such as CREB (cAMP-response element binding protein), which help orchestrate many cellular processes including neurite outgrowth, synaptic plasticity, long-term potentiation, and survival (Patapoutian & Reichardt, 2001).

Phosphorylation of the Shc site in TrkB also mediates activation of PI-3K signaling via the Ras or GAB-1 family of adaptor proteins. Under basal conditions, PI-3K activity has been identified as the dominant pathway of BDNF-stimulated cell survival (Hetman, Kanning, Cavanaugh, & Xia, 1999). PI-3K activation targets Akt, which promotes cell survival by inhibiting pro-apoptotic factors such as the forkhead transcription factor, Bcl-2-associated death promoter (BAD) protein, and p53 (Brunet, Datta, & Greenberg, 2001; Datta et al., 1997).

A third major pathway stimulated by TrkB activation is PLC-γ. Tyrosine phosphorylation at position Y816 on TrkB and analogous residues in TrkA and TrkC recruits and activates the enzyme PLC-γ which catalyzes the breakdown of lipids to produce diacyglycerol (DAG) and inositol-3-phosphate (IP3). Whereas DAG allows for maximal

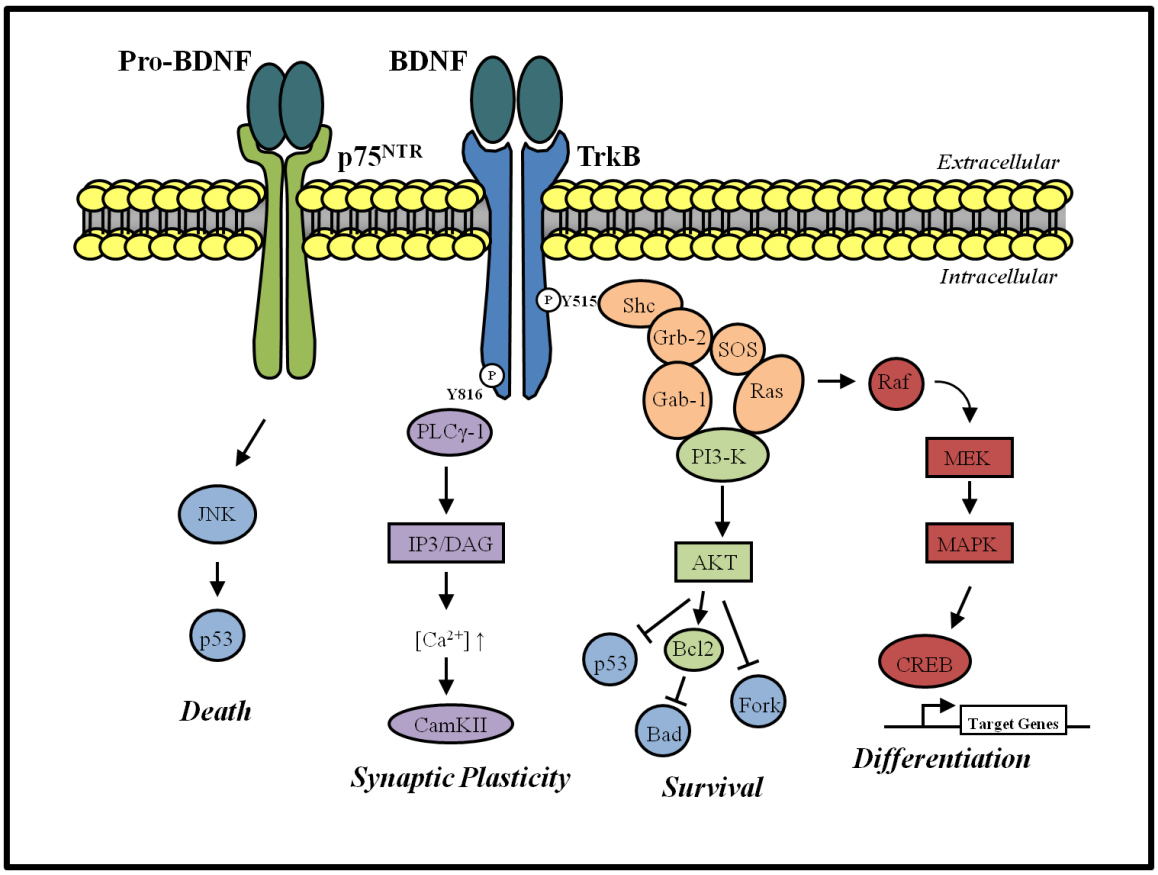

Figure 1.1

P75^{NTR} and TrkB receptors mediate the biological responses of BDNF through distinct signal transduction pathways. BDNF binds TrkB with high affinity to induce receptor dimerization and autophosphorylation of tyrosine residues which serve to activate PLCγ, PI3K and MAPK cascades and support synaptic plasticity, survival, and differentiation of neurons, respectively. Pro-BDNF binds p75^{NTR} leading to apoptosis through the JNK cascade.

activation of protein kinase C (PKC), IP3 promotes release of Ca^{2+} from internal stores (Segal, 2003). This increases activity of synaptic CaM kinases and may potentiate neurotransmitter release, which suggests that this pathway may regulate excitatory synaptic plasticity. Consistent with this notion, LTP in the hippocampus is significantly impaired in TrkB mice with a PLC-γ binding site mutation (Minichiello et al., 2002).

$P75^{NTR}$ Signaling

The P75 neurotrophin receptor was the first neurotrophin receptor to be isolated, but our understanding of its functional role has lagged behind that of Trk receptors. $P75^{NTR}$ is expressed developmentally and signaling through this receptor can contribute to pruning of developing neuronal populations (via pro-BDNF). All neurotrophins can bind $P75^{NTR}$ with similar affinity however, pro-neurotrophins bind with much higher affinity and mediate the majority of $P75^{NTR}$ function, that is cell death (Rodriguez-Tebar, Dechant, & Barde, 1991).

$P75^{NTR}$ is composed of a cysteine-rich extracellular domain with a single transmembrane domain and, unlike Trks, lacks an intracellular kinase domain. Instead, it possesses an intracellular "death" domain homologous to that of the tumor necrosis factor-α (TNFα) receptor, allowing $P75^{NTR}$ to signal as an independent receptor. Activation of $P75^{NTR}$ by pro-BDNF causes sphingomyelin hydrolysis (Dobrowsky, Werner, Castellino, Chao, & Hannun, 1994; Dobrowsky, Jenkins, & Hannun, 1995), enhancement of c-Jun *N*-terminal kinase (JNK), and activity of the tumor suppressor gene p53, all events which are linked to cell death (Fig 1.1) (Aloyz et al., 1998; Bamji et al., 1998).

Many cells co-express Trk and $p75^{NTR}$, and this pattern of expression has a functional significance. Co-immunoprecipitation studies indicate that physical complexes form between $P75^{NTR}$ and Trk (Bibel, Hoppe, & Barde, 1999). Moreover, $P75^{NTR}$ may induce conformational changes in Trk extracellular domains to alter the ligand binding site, thereby regulating the affinity of neurotrophins to Trk receptors (Zaccaro, Ivanisevic, Perez, Meakin, & Saragovi, 2001). In summary, TrkB and $P75^{NTR}$ receptors are integral components in the signaling pathways facilitating BDNFs diverse functions in the brain not the least of which includes the control of food intake and body weight.

BDNF Signaling Regulates Food Intake and Body Weight

The increasing global prevalence of obesity and its associated medical complications including type-2 diabetes and cardiovascular disease highlights the need to understand the mechanisms underlying feeding behavior. According to the world health organization, nearly 60-70% of US adults are overweight or obese. Moreover, in 2010, 43 million children <5 years of age were overweight (de Onis, Blossner, & Borghi, 2010). While dietary and environmental factors have undoubtedly contributed to the current obesity trends, inheritable biological factors that disrupt the tightly regulated equilibrium between caloric intake and energy expenditure also play a critical part. Mounting evidence from rodent and human studies suggests that perturbed BDNF and TrkB signaling in appetite-regulating centers of the brain is such a biological risk factor for obesity.

The first report suggesting a role for BDNF in food intake regulation was made 20 years ago, when Lapchak and Hefti demonstrated that central infusion of BDNF attenuated

weight gain in rats (Lapchak & Hefti, 1992) by dose dependently decreasing food intake (Pelleymounter, Cullen, & Wellman, 1995). More recent studies investigating the role of BDNF on food intake have used genetic tools to inactivate BDNF or its receptor, TrkB. BDNF (+/-) mice carrying one functional *Bdnf* allele, express 50% of normal BDNF levels and exhibit increased food intake and develop obesity (Kernie, Liebl, & Parada, 2000; Lyons et al., 1999). Conditional mutant mice with central depletion of BDNF ($BDNF^{2L/2LCk\text{-}cre}$ mice) consume nearly twice as many calories as wild type controls (Rios et al., 2001). In addition to 80% and 150% increases in body weight among respective male and female mutants, central BDNF depletion also leads to other metabolic defects including leptin and insulin resistance, dyslipidaemia and hyperglycemia (Rios et al., 2001). These metabolic alterations are secondary to the obesity because limiting the caloric intake to wild type levels (pair feeding) is sufficient to restore normal body weight and normalize these metabolic parameters (Coppola & Tessarollo, 2004). Lastly, TrkB hypomorphic animals in which full length TrkB is only expressed at about 25% of normal levels, display hyperphagia and obesity (Xu et al., 2003). Pharmacological studies have shown that TrkB agonists (including BDNF and NT4) suppress appetite and body weight in addition to long lasting amelioration of hyperglycemia (Tsao et al., 2008). Together, the cumulative evidence clearly implicates a critical role for BDNF and TrkB signaling in food intake and body weight regulation in rodents.

Several human studies have also linked BDNF and TrkB to obesity and eating disorders. Yeo et al (2004) described an 8 year old male patient heterozygous for a *de novo* missense mutation in TrkB (impeding phosphorylation and activation of MAPK), who exhibited hyperphagic behavior and severe obesity (Yeo et al., 2004). Similarly,

haploinsufficiency for BDNF by means of a chromosomal inversion encompassing *Bdnf* associated with decreased serum levels of BDNF, severe hyperphagia, and obesity in an 8 year old female patient (J. Gray, Yeo, Cox, Morton, Adlam, Keogh, Yanovski, El Gharbawy, Han, Tung, Hodges, Raymond, O'rahilly, & Farooqi, 2006a). Moreover, BDNF haploinsufficiency in Wilms tumor, aniridia, genitourinary anomalies, and mental retardation (WAGR) syndrome as a result of *Bdnf* deletions is also associated with hyperphagia and childhood-onset obesity (Han et al., 2008). Finally, a recent association meta-analysis of nearly 250,000 individuals identified *Bdnf* as one of a handful of genetic loci linked to obesity susceptibility in humans (Speliotes et al., 2010).

Extensive evidence suggests that the Val66Met *Bdnf* polymorphism, by significantly lowering serum levels of BDNF, may genetically predispose humans to severe binge eating behavior leading to obesity (Bhang, Ahn, & Choi, 2011). This is particularly relevant given that 27% and 4.5% of American individuals have been estimated to be heterozygous and homozygous carriers of the polymorphism, respectively (Shimizu, Hashimoto, & Iyo, 2004). The frequency of the Met allele is significantly associated with an increase in BMI obesity susceptibility in children (Skledar et al., 2012). Furthermore, a recent meta-analysis concluded that Met allele carriers have a 36% higher risk of developing eating disorders than Val/Val patients (Gratacos et al., 2007). In agreement with this, Koizumi et al (2004) identified an increase in the Val66Met genotype frequency in a cohort of Japanese females with bulimia nervosa (BN) (Koizumi et al., 2004). Additionally, in a population of Caucasian females diagnosed with either BN or binge eating disorder (BED), the Val66Met polymorphism was associated with increased weekly frequency of bingeing and severity of binging episodes (Monteleone et al., 2006). Finally, it was reported that the BDNF Met

allele contributes to a higher risk of binge eating in women with a history of dietary food restriction (Akkermann, Hiio, Villa, & Harro, 2011). Evidence emerging from these rodent and human studies clearly validates the importance of BDNF signaling in the central nervous system (CNS) for the regulation of food intake and eating behavior. However, the underlying cellular and molecular mechanisms remain poorly defined and are the focus of this thesis.

BDNF and the Central Mechanisms of Food Intake Regulation

Food intake is a complex behavior coordinated in the brain not only by homeostatic mechanisms balancing nutritional requirements and caloric status but also by hedonic factors that regulate the sense of pleasure and reward derived from consuming palatable foods. Whereas homeostatic mechanisms are regulated primarily within the hypothalamus and dorsal vagal complex (DVC), hedonic pleasure derived from indulging in palatable food is thought to be mediated by a midbrain circuit known as the mesolimbic dopamine pathway. These neural substrates constitute distinct yet interrelated pathways mediating food intake behavior, the underlying workings of which are not completely understood. A wide body of evidence, however, has established pivotal roles for BDNF and its receptor, TrkB, in food intake, energy homeostasis, and hedonic eating behavior leading to obesity. Evidence for BDNF and TrkB participation in the regulation of both homeostatic and hedonic feeding by acting in energy balance and reward centers in the brain are reviewed here.

BDNF and Energy Homeostasis

The hypothalamus is located ventral to the thalamus along the walls of the third ventricle. By receiving and processing peripheral metabolic signals of satiety, hunger, and adiposity, the hypothalamus is a key integrative center in the brain serving to maintain energy homeostasis; balancing caloric intake and energy expenditure. In response to metabolic cues, neurons of the hypothalamus modify the expression and action of select orexigenic or anorexigenic neuropeptides within interconnected hypothalamic nuclei and coordinate the appropriate behavioral responses to stimulate or inhibit feeding, respectively. During conditions of positive energy balance, high circulating levels of satiety factors including glucose, the adipocyte-derived leptin, gut-derived peptide YY (PYY) and cholesystokinin (CCK), and insulin from the pancreas increase the anorexigenic tone in the hypothalamus to inhibit feeding. Conversely, when energy levels are depleted, low levels of satiety factors and increased secretion of the orexigenic factor ghrelin, emanating from the stomach, enhance orexigenic signaling to stimulate feeding.

The major hypothalamic nuclei involved in this homeostatic circuitry include the arcuate nucleus (Arc), ventromedial nucleus (VMH), dorsomedial nucleus (DMH), paraventricular nucleus (PVN), and the lateral nucleus (LH) (Fig 1.2). Major studies examining food intake regulation with particular regard to the role of BDNF/TrkB signaling within these anatomical substrates are discussed here.

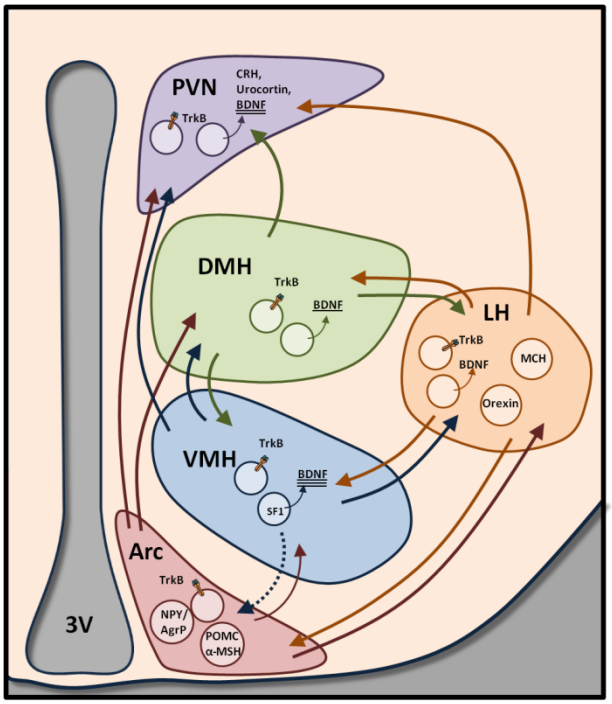

Figure 1.2

Hypothalamic anatomy involved in homeostatic appetite control. This simplified diagram illustrates the connectivity between hypothalamic nuclei controlling food intake, in a manner that emphasizes BDNF signaling. Some additional signaling pathways are left out to preserve clarity. Arc neurons produce either POMC, the precursor for α-MSH, or NPY and AgrP. These cells display widespread interconnectivity (denoted by arrows) with other nuclei including the PVN, DMH, and LH to coordinate control of homeostatic food intake. During periods of positive energy balance, Arc POMC neurons are dynamically targeted (dashed line) by the VMH. The VMH and PVN express the highest BDNF levels in the hypothalamus (underlined according to relative expression), and have well established roles mediating satiety through TrkB. In contrast, BDNF and TrkB are minimally expressed in the LH and are unlikely to influence feeding via Orexin and MCH.

Arcuate Nucleus

The Arc is located in the mediobasal portion of the hypothalamus immediately superior to the median eminence and is considered a primary nutrient-sensing center of the hypothalamus. It contains two distinct populations of neurons that exert opposing effects on food intake and energy balance. The proximity of both neuronal systems to the median eminence, a region with no blood brain barrier, as well as interaction with local capillaries allows for effective targeting of the Arc by peripheral metabolic signals (Cone et al., 2001).

Anorexigenic neurons in the Arc synthesize pro-opiomelanocortin (POMC), a precursor for α-melanocyte stimulating hormone (α-MSH). Activation of POMC neurons by satiety signals such as leptin promotes the release of α-MSH from POMC axon terminals onto target sites where it activates melanocortin receptor 3 and 4 (MC3R and MC4R) to suppress food intake and increase metabolism (Mizuno et al., 1998). In support of this anorexigenic action, mice lacking either MC3R or MC4R are obese (Butler et al., 2000; Huszar et al., 1997). Moreover, light activation of POMC neurons using the cation channel channelrhodopsin (ChR2) reduces food intake and body weight in a MCR dependent manner (Aponte, Atasoy, & Sternson, 2011). POMC neurons also express cocaine-and amphetamine-regulated transcript (CART), an anorectic peptide (Kristensen et al., 1998) which may exert satiety via the hypothalamic-pituitary-adrenal (HPA) axis (Vrang, Larsen, Kristensen, & Tang-Christensen, 2000). However, blocking MCRs prevents hypophagia mediated by POMC neuronal activity, arguing that CART is not sufficient to reduce food intake (Aponte et al., 2011).

Orexigenic neurons in the Arc express neuropeptide Y (NPY). Most NPY neurons (95%) also express agouti-related protein (AgrP), an endogenous antagonist of MC4R (Broberger, Johansen, Johansson, Schalling, & Hokfelt, 1998; Ollmann et al., 1997). Delivery of NPY or AgrP to the brain (Clark, Kalra, & Kalra, 1985; C. J. Small et al., 2001) or activation of NPY/AgrP neurons using ChR2 technology in the Arc stimulates voracious feeding in mice, evidence strongly supporting an orexigenic role for this cell population (Aponte et al., 2011). Activation of NPY/AgrP neurons by ghrelin promotes feeding by stimulating the release of NPY and AgrP, antagonizing the anorexigenic effect of α -MSH at MC4R (Ollmann et al., 1997), and inhibiting POMC neurons by direct GABA innervations (Cowley et al., 2001). This unidirectional interaction between NPY/Agrp inputs onto POMC neurons may represent a circuitry evolutionarily wired to favor the inhibition of satiety signals to promote feeding, as well as overfeeding when food is available in excess (Gao & Horvath, 2008).

The POMC/CART and NPY/AgrP neurons in the Arc provide projections to other hypothalamic nuclei expressing NPY and MC4R receptors including the PVN, VMH, DMH, and LH (Mountjoy, Mortrud, Low, Simerly, & Cone, 1994; Parker & Herzog, 1999; Xu et al., 2003). The Arc also sends extra-hypothalamic projections neurons in the NTS (Bagnol et al., 1999). Little is known regarding the role of TrkB signaling in the Arc. This region lacks BDNF expression but contains TrkB (Yan et al., 1997). Considering the well demonstrated role of BDNF in synaptic plasticity in the brain (Lu & Figurov, 1997), it is plausible that it participates in synaptic remodeling processes known to occur in the Arc. Indeed, the strength of the excitatory input from the medial VMH to POMC neurons is diminished by 24 hours of fasting and restored by refeeding (Sternson, Shepherd, & Friedman, 2005). Future

investigations should assess whether BDNF signaling plays a role in this functional plasticity to influence feeding behavior.

Paraventricular Nucleus

The PVN is located in the rostral hypothalamus surrounding the roof of the third ventricle and is an important integration center for melanocortin signal originating from the Arc. The PVN receives dense POMC/CART and NPY/AgrP afferents from the Arc and makes reciprocal connections with the DMH, VMH, LH and DVC (Ranson, Motawei, Pyner, & Coote, 1998). Several neuropeptides affect caloric intake and energy metabolism by signaling in the PVN. NPY and AgrP administration to the PVN, for example, stimulate feeding and decrease energy expenditure without influencing physical activity (Stanley & Leibowitz, 1984; Tang-Christensen et al., 2004; van Dijk & Strubbe, 2003; Wirth & Giraudo, 2000). In contrast, leptin administration significantly reduces energy intake and enhances energy expenditure (Bagnasco, Dube, Katz, Kalra, & Kalra, 2003).

Regulation of food intake and energy expenditure in the PVN is also mediated by BDNF which, like TrkB, is highly expressed in this hypothalamic region (Kernie et al., 2000). Accordingly, acute injection of BDNF in the PVN reduces body weight by decreasing feeding and increasing energy expenditure in rats (C. Wang, Bomberg, Billington, Levine, & Kotz, 2007a; C. Wang, Bomberg, Billington, Levine, & Kotz, 2007b). Chronic BDNF administration to the PVN is also sufficient to reduce HFD-induced obesity and its associated metabolic defects including high serum levels of glucose, insulin, and leptin (C. Wang, Godar, Billington, & Kotz, 2010). One possible mechanism by which BDNF in the PVN

affects feeding and energy expenditure is via the corticotrophin-releasing hormone (CRH) pathway. CRH and its related family member, urocortin, strongly inhibit food intake and stimulate energy expenditure (Richard, Huang, & Timofeeva, 2000; Spina et al., 1996). Chronic intra-cerebroventricular (i.c.v.) or intra-PVN administration of BDNF promotes the upregulation of CRH and urocortin in PVN neurons containing TrkB. Notably, co-administration of α-helical-CRH, an antagonist for the CRH and urocortin receptors CRH-R1 and R2, counteracted the effects of BDNF. Selective blockade of CRH-R2, the high affinity urocortin receptor, was also sufficient to inhibit BDNF-induced anorexia (Toriya et al., 2010). Another mechanism explaining BDNF's anorexigenic actions in the PVN involves remodeling inhibitory synaptic contacts. By reducing the surface expression of post-synaptic $GABA_A$ receptors (Hewitt & Bains, 2006), BDNF can minimize feeding responses elicited by NPY-GABA co-action in the PVN (S. Pu et al., 1999). Together, these data identify the PVN as an important hypothalamic region mediating the long term effects of BDNF to reduce food intake.

Ventromedial Nucleus

The VMH is a cell group located in the mediobasal hypothalamus, adjacent to the third ventricle and superior to the Arc. Receiving sparse input from α-MSH fibers originating in the Arc, the VMH, for its part, sends glutamatergic, excitatory projections to Arc POMC neurons, forming a circuit which is dynamically regulated by nutritional state (Sternson et al., 2005). VMH neurons also make reciprocal connections with the PVN, DMH, and LH. Minimal outputs to the amygdala and nucleus accumbens (Canteras,

Simerly, & Swanson, 1994) suggest a potential link between homeostatic and hedonic feeding circuits in the brain.

The VMH has long been recognized as a "satiety center" in the brain, playing a crucial role in energy balance regulation. Hetherington and Ranson (1940) demonstrated that bilateral electrolytic lesions of the rat VMH resulted in hyperphagia and obesity (Hetherington & Ranson 1940). It was further suggested that VMH lesions also reduce energy expenditure, since the obesity observed in VMH lesioned animals persisted even when caloric intake was restricted to control levels (Cox & Powley, 1981). More recently, the VMH has been identified to serve as a critical center for the integration of nutritional cues including glucose, orexin, insulin, and leptin, although the identity of these nutrient-sensing neurons has not been established (McClellan, Parker, & Tobet, 2006).

Of all the hypothalamic nuclei, BDNF expression is highest in the VMH (Xu et al., 2003). Several studies indicate an essential role for BDNF in appetite-suppressing processes acting in this region. In support of this, BDNF and TrkB expression in the VMH is induced by positive energy balance and repressed in the fasted state (Komori, Morikawa, Nanjo, & Senba, 2006; Unger, Calderon, Bradley, Sena-Esteves, & Rios, 2007; Xu et al., 2003). Moreover, long-term infusion of BDNF in the VMH decreases feeding, increases energy expenditure (resting metabolic rate and locomotor activity) and decreases body weight (C. Wang, Bomberg, Billington, Levine, & Kotz, 2007a; C. Wang, Bomberg, Levine, Billington, & Kotz, 2007; C. Wang et al., 2010). Finally, systemic administration of glucose, a caloric signal, selectively induces the transcription and expression of BDNF in the VMH by action of promoter I (Unger et al., 2007).

In addition to glucose, there are several transcriptional regulators of BDNF which support BDNFs anorexigenic actions in the VMH. For example, leptin induces BDNF mRNA and protein expression here (Komori et al., 2006). Moreover, the nuclear receptor NR5A1 known as steroidogenic factor 1 (SF-1), is a transcription factor expressed exclusively in the VMH which mediates *Bdnf* exon specific transcripts I, II, and IV (Tran, Akana, Malkovska, Dallman, Parada, & Ingraham, 2006a). SF-1 heterozygous mice display decreased BDNF (Tran, Akana, Malkovska, Dallman, Parada, & Ingraham, 2006a) and are obese (Majdic et al., 2002). Furthermore, RAI1 is a novel gene which acts as a transcriptional activator of *Bdnf* via an enhancer element in Bdnf intron I in the hypothalamus (Burns et al., 2010). RAI1 happloinsufficiency decreases BDNF and promotes hyperphagia and obesity in mice and humans (Burns et al., 2010). The necessity for BDNF signaling in the VMH was illustrated by work in our lab demonstrating that selective *Bdnf* deletion in the adult VMH elicits hyperphagia and obesity (Unger et al., 2007). Altogether, this evidence strongly supports a model in which BDNF acts as a satiety factor in the VMH.

BDNF appears to regulate feeding downstream of POMC signaling in the VMH. MC4R stimulation induces BDNF expression here and administration of BDNF suppresses the hyperphagia and weight gain observed in MC4R-/- mice (Xu et al., 2003). Similarly, the hyperphagic and obese phenotype in agouti lethal yellow mice (Ay), which have disrupted MC4R signaling as a result of ectopic expression of agouti protein (a homologue of AgrP), can also be reversed by infusion of BDNF (Nakagawa et al., 2003). Intra-VMH infusion of BDNF also significantly inhibits NPY-induced feeding and body weight gain via TrkB signaling (C. Wang, Bomberg, Levine et al., 2007). The anorexigenic actions of BDNF in the VMH are well illustrated. However, the mechanisms downstream of BDNF mediating its

satiety effects in the VMH remain to be defined and are the focus of work described in this thesis.

Dorsomedial Nucleus

The DMH is located immediately superior to the VMH, a nucleus with which it shares reciprocal connectivity (Luiten & Room, 1980). The DMH is extensively innervated by the Arc and projects to other intra and extra-hypothalamic nuclei known for regulating food intake including the PVN, LH, and DVC. A role of the DMH in the modulation in energy intake is supported by the finding that destruction of the DMH results in hypophagia and reduced body weight in rats (Bellinger, Bernardis, & Brooks, 1979). Moreover, direct NPY administration promotes food intake (L. Yang et al., 2009). Decreased feeding as a result of CCK and MC4R agonist administration in the DMH indicates that anorexigenic systems are also present in this nucleus (Blevins, Stanley, & Reidelberger, 2000; P. Chen, Williams, Grove, & Smith, 2004). Because the DMH also receives input from the suprachiasmatic nucleus, a circadian pacemaker, it has been further suggested that the DMH conveys circadian influence on food intake. Indeed, DMH lesions disrupt circadian distribution of feeding and arousal (Chou et al., 2003; Gooley, Schomer, & Saper, 2006), an effect which may be mediated via glutamatergic projections to the PVN, and LH (ter Horst & Luiten, 1986). Both BDNF and TrkB are expressed in the DMH at lower levels than the VMH (Xu et al., 2003; Yan et al., 1997) but their role here has been surprisingly unstudied.

Lateral Hypothalamus

The LH, a heterogeneous area in the lateral zone of the hypothalamus, was first recognized as an important feeding center in the brain based on lesion studies that induced anorexia in rats (Anand & Brobeck, 1951). It contains two unique neuronal populations: one group synthesizes orexin (also known as hypocretin), a peptide implicated in arousal, and the other synthesizes melanin-concentrating hormone (MCH) (Qu et al., 1996; Sakurai et al., 1998). Both neuronal populations promote feeding. Whereas MCH knockout mice are hypophagic and lean, MCH overexpressing mice are obese (Ludwig et al., 2001; Shimada, Tritos, Lowell, Flier, & Maratos-Flier, 1998). While orexin knockout mice exhibit normal food intake, they display decreased sucrose consumption (Matsuo et al., 2011). ICV administration of orexin promotes standard and palatable food intake (Sakurai et al., 1998; Thorpe, Cleary, Levine, & Kotz, 2005) as well as sucrose consumption (Baird et al., 2009).

The orexigenic effects of the LH are thought to be mediated via bidirectional interactions with hypothalamic nuclei involved in homesostatic control of food intake, namely the Arc, PVN, VMH, and DMH as well as projections to the mesolimbic circuit involved in hedonic feeding (Bittencourt, Arruda, de Andrade, & Carvalho, 1991; Broberger et al., 1998). Orexins, for example, promote food intake by directly exciting NPY neurons and inhibiting POMC neurons in the Arc (Muroya et al., 2004). Similarly, increased feeding is observed in rats following MCH injections in the Arc, PVN, and to a lesser extent the DMH (but not the VMH) (Abbott et al., 2003).

While the exact mechanisms by which LH neurons drive feeding are not known, the projections of MCH and orexin neurons from the LH are far reaching, suggesting multiple

levels of feeding regulation. MCH and Orexin act in the nucleus accumbens (NAc) and ventral tegmental area (VTA) to modulate hedonic feeding (Georgescu et al., 2005). Major targets of the LH neurons also include brain-stem motor systems supporting chewing, licking, and swallowing behavior (Lund, Kolta, Westberg, & Scott, 1998). Low levels of BDNF and TrkB are present in the LH and display very little coexpression with MCH or orexin (Xu et al., 2003; Yan et al., 1997). Consistent with this, acute BDNF delivery to the LH does not influence feeding behavior in rats (C. Wang, Bomberg, Billington, Levine, & Kotz, 2007a). It is therefore unlikely that BDNF regulates food intake by directly modulating these neurons.

Dorsal Vagal Complex

The DVC is located in the caudal brainstem and is comprised of three main structures including the nucleus of the solitary tract (NTS), the area postrema, and the dorsal motor nucleus of the vagus nerve (DMX). The DVC serves as the primary sensory site for visceral and taste input in the brainstem. Not surprisingly, its ascending connectivity with the hypothalamus is important in the control of food intake. Efferent projections from the DVC have been demonstrated to all hypothalamic nuclei which are involved in regulation of feeding and body weight, with the exception of the VMH (Ter Horst, de Boer, Luiten, & van Willigen, 1989). The DVC expresses both BDNF and TrkB (Conner, Lauterborn, Yan, Gall, & Varon, 1997), suggesting that it is another site for the anorexigenic effects of BDNF. Accordingly, exogenous application of BDNF in the DVC induces hypophagia and weight loss. Moreover, endogenous BDNF content here is directly correlated to nutritional state

and levels of satiety factors including leptin and CCK (Bariohay, Lebrun, Moyse, & Jean, 2005). The orexigenic effects of MC3/4R antagonism within the DVC, can also be blocked by co-administration of BDNF (Bariohay et al., 2009). Together, this supports a role for BDNF acting downstream of melanocortin signaling within the DVC to regulate food intake.

A Mechanism Mediating the Satiety Effects of BDNF in the Hypothalamus

Synaptic circuits within the hypothalamus are dynamically regulated according to nutritional status and metabolic signals. Leptin, for example, increases excitatory input onto POMC neurons in the Arc, reversing the synaptic profile during positive energy balance (Pinto et al., 2004; Sternson et al., 2005). Thus, synaptogenesis and plasticity between hypothalamic nuclei represents a mechanism thought to mediate homeostatic food intake regulation. In this thesis, I introduce α2δ-1, which has the capacity to promote excitatory synapse formation, as a novel and critical component within this appetitive circuitry acting downstream of BDNF (Christopherson et al., 2005; Eroglu et al., 2009). My experiments demonstrating its role in this context will be described in detail in Chapter 2. Here, I provide introductory material supporting our motivation to examine the effects of α2δ-1 facilitating satiety in the hypothalamus.

α2δ-1, is an accessory subunit of high voltage-gated Ca^{2+} (Ca_V) channels, which are required for calcium-dependent neurotransmitter release at synapses in the central nervous system. In addition to α2δ-1, the heteromeric complex forming a Ca_V channel includes α1 and β subunits. The α1 subunit, which provides the ion-conducting pore of Ca_V, directly interacts and co-purifies with β and α2δ auxiliary subunits (Dooley, Taylor, Donevan, &

Feltner, 2007). Together, β and α2δ subunits are primarily thought to mediate $Ca_V\alpha1$ trafficking and activity (Fig 1.3) (Jarvis & Zamponi, 2007).

The accessory α2δ-1 subunit is translated from a single gene, which is post-translationaly cleaved into an extracellular α2 moiety with a membrane-bound δ-1 component. The α2 and the δ-1 peptides associate by disulfide bridges that form between cysteine residues found in both peptides (Marais, Klugbauer, & Hofmann, 2001). Alternative splicing produces at least 5 isoforms of α2δ-1 ($\alpha2\delta\text{-}1_{A\text{-}E}$), each following a tissue specific pattern of expression, where only $\alpha2\delta\text{-}1_B$ is specific to the brain. Within the brain, α2δ-1 is highly expressed in glutamatergic neurons of the VMH, PVN, and DVC, key nuclei involved in BDNF-mediated satiety (Cole et al., 2005). These splice variants have not been fully characterized and the functional significance of their expression pattern have yet to be established (Angelotti & Hofmann, 1996). However, the primary function attributed to α2δ-1 involves the enhancement of calcium channel activity by directly interacting with and increasing trafficking of the pore forming subunit of the calcium channel, $Ca_V\alpha1$, to the plasma membrane (Dooley et al., 2007).

The trafficking properties of α2δ-1 are regulated by a von Willebrand factor type A (VWA) domain (common to all α2δ subunits) located on the extracellular, α2 moiety. Within the VWA domain, a metal ion adhesion site (MIDAS) motif, which confers divalent-cation-dependent interactions between the VWA domain and its ligands, is essential for the trafficking function of α2δ-1. Mutations within the MIDAS motif, for example, prevent the α2δ-1 mediated enhancement of Ca^{2+} current at the plasma membrane by increasing intracellular retention of $Ca_V\alpha1$ (Canti et al., 2005). The mechanism of trafficking is not clear, but it is likely that α2δ-1 subunits associate with α1 subunits in the endoplasmic

reticulum and the resulting complex is transported to the plasma membrane (Gurnett, Felix, & Campbell, 1997).

Adjacent (N-terminal) to the VWA domain is a RRR motif necessary for the binding of Gabapentin (GP) and Pregabalin (PGB), anti-neuropathic pain and anti-epileptic drugs (M. Wang, Offord, Oxender, & Su, 1999). These drugs, which are transported inside the cell via L -amino acid transporters, act primarily at intracellular locations whereby chronic but not acute pharmacological application inhibits the trafficking and cell surface expression of both α2δ-1 and α1 (Heblich, Tran Van Minh, Hendrich, Watschinger, & Dolphin, 2008; Hendrich et al., 2008). This has the effect of preventing the enhancement Ca^{2+} currents mediated by α2δ-1, outlining a mechanism thought to mediate the therapeutic action of gabapentinoid drugs (Field et al., 2006). Patients administered GP and PGB display several negative side effects, most notably including increased appetite and severe body weight gain (DeToledo, Toledo, DeCerce, & Ramsay, 1997; Hoppe, Rademacher, Hoffmann, Schmidt, & Elger, 2008), suggesting that their pharmaceutical target, α2δ-1, might facilitate mechanisms of appetite control.

Exactly how GP exerts these effects at the molecular level remains to be fully elucidated. It is known that α2δ-1 surface expression inhibition by GP takes place by a decrease in forward trafficking from the Golgi rather than an increase in endocytosis (Bauer et al., 2009). Moreover it was proposed that endogenous ligand binding to the VWA domain induces a conformational change that favors trafficking (Canti et al., 2005; Eroglu et al., 2009). GP binding to α2δ-1 may restrict access to the VWA domain, keeping α2δ-1 in its "inactive conformation," preventing trafficking or binding of endogenous ligands. Supportive is the demonstration that GP disrupts the binding interaction between the VWA

domain and known ligands including thrombospondins (TSPs) (Eroglu et al., 2009). It is thus interesting to speculate that intracellular ligands which compete for GP for binding, including GABA, leucine, and isoleucine (Dooley et al., 2007), may also serve as endogenous regulators for α2δ-1 trafficking.

Much research has focused on the role of α2δ-1 in the regulation of Ca_V function and trafficking. However, evidence by Eroglu et al., imply that at least some novel roles for α2δ-1 involve extracellular signaling via VWA domains. In particular, they demonstrate that TSPs bind to the VWA domain of α2δ-1 to promote excitatory synaptogenesis in the CNS (Christopherson et al., 2005; Eroglu et al., 2009). Because the application of Ca^{2+} channel blockers does not influence synapse formation, α2δ-1 mediated synaptogenesis does not depend on calcium channel expression or function (Eroglu et al., 2009).

TSPs make up a family of secreted extracellular matrix proteins which are highly expressed in the brain and periphery. TSP1, the first of the 5 presently known TSPs to be cloned, was initially identified as platelet-derived factor with pro-thrombotic properties (Christopherson et al., 2005). However, diverse signaling functions for TSP isoforms including cell adhesion, migration, and neurite outgrowth have also been defined, largely according to their structural signaling features. The EGF-like repeats common to all TSP isotypes are essential to promoting synaptogenesis by interaction with the VWA domain of α2δ-1 subunits (Christopherson et al., 2005; Eroglu et al., 2009).

Expression profiling of different cell types in the brain show that mRNA for TSP is particularly enriched in developing astrocytes (Christopherson et al., 2005). Select populations of neurons also express TSP in the adult brain, implying that neuron-derived TSP

in the adult brain may also serve important roles in synapse formation and plasticity (Arber & Caroni, 1995). Consistent with this, TSP expression dramatically decreases with maturity in the adult rodent brain and becomes restricted to regions of neurogenesis and plasticity (Hoffman, Dixit, & O'Shea, 1994). Secretion by either neurons or astrocytes allows for TSP incorporation in the extracellular matrix, where it may interact with other proteins such as α2δ-1 (Arber & Caroni, 1995). Evidence also suggests that TSPs and α2δ-1 might interact intracellularly during trafficking since TSP4 and α2δ-1 mRNA are concomitantly upregulated in the DRG in experimental models for neuropathic pain (H. Wang et al., 2002).

The cumulative data suggest that the synaptogenic interaction between α2δ-1 and TSPs in the adult brain underlie synaptic remodeling between hypothalamic nuclei. Because of BDNF's well recognized role in synaptic plasticity, α2δ-1 interactions with TSP may also represent a mechanism through which BDNF maintains control of homeostatic food intake regulation. I describe work testing this hypothesis in this thesis.

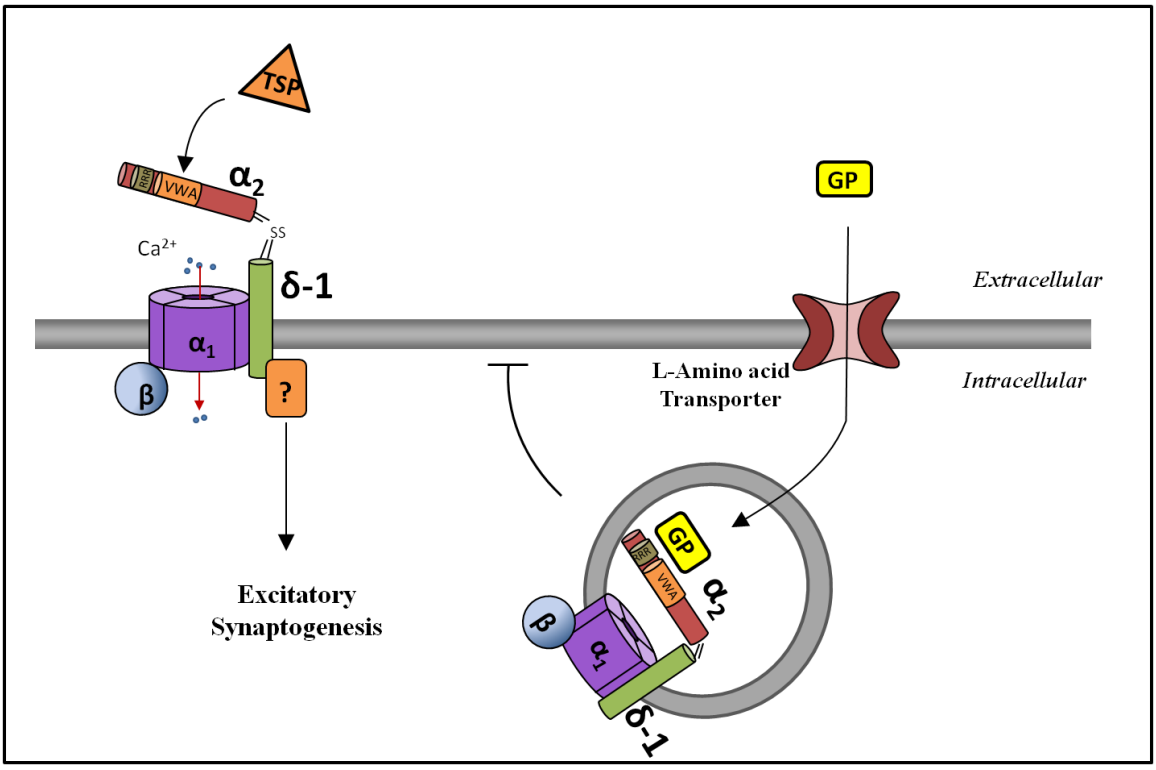

Figure 1.3

α2δ-1 is a neuronal thrombospondin and gabapentin receptor. α2δ-1 and β subunits enhance trafficking of the high-voltage calcium channel complex (CaV) to the cell membrane, which allows Ca^{2+} influx via the pore forming α1 subunit. Endogenous thrombospodin (TSP) can bind to the von Willebrand factor type A (VWA) domain on the extracellular α2 moiety of α2δ-1 to mediate excitatory synaptogenesis independent of calcium channel function, albeit through an undefined signaling partner. Gabapentin (GP) is transported to the cytoplasm via the L-amino acid transporter where it acts intracellularly to bind an RRR motif on α2δ-1 and inhibit trafficking of the Ca_V complex to the surface, thereby reducing whole-cell Ca^{2+} current and inhibiting TSP-mediated synaptogenesis.

BDNF and Hedonic Feeding Control in the Mesolimbic Dopamine System

Food intake is not only driven by homeostatic mechanisms balancing satiety signals and nutritional requirements. The sense of pleasure or reward derived from consuming highly palatable food can often override homeostasis and promote consumption in excess of metabolic need. Well-fed rats with free access to standard chow, for example, will voluntarily expose themselves to extreme cold (-15 °C), noxious heat pain, or aversive foot shock to obtain shortcake, meat pate, peanut butter, Coca-cola, chocolate chips, and yogurt drops (Cabanac & Johnson, 1983; Foo & Mason, 2005; Oswald, Murdaugh, King, & Boggiano, 2011). These palatable food items promote excessive hedonic feeding by activating a midbrain circuit known as the mesolimbic dopamine system.

The mesolimbic dopamine system has long been recognized as one of the critical anatomical substrates orchestrating the dopamine-dependent behavioral effects to drugs of abuse and natural rewards such as food. It is composed of dopamine (DA) neurons that originate in the ventral tegmental area (VTA) and project to the nucleus accumbens (NAc) as well as the prefrontal cortex (PFC) (Fig 1.4) (Ungerstedt, 1971). Similar to drugs of abuse, palatable food promotes synaptic release of DA from the VTA onto NAc and PFC targets (Bassareo & Di Chiara, 1999) which forms the basis for the model in which DA signaling regulates the incentive value, reward desire, and motivation to pursue highly palatable food.

As reviewed herein, extremely precise anatomical relationships exist between the NAc, VTA, and PFC and their projection targets, altogether indicating that the motivation to consume palatable food intake and the reward value associated with it are very tightly regulated within the mesolimbic circuitry. Notably, recent evidence suggests BDNF

signaling may modulate behavioral plasticity within this circuitry, which is of particular relevance since dysregulation of dopamine transmission here has been linked to excessive hedonic eating (Geiger et al., 2009).

Ventral Tegmental Area

The VTA is a group of neurons located along the floor of the midbrain, medial to the substantia nigra. The primary cell type in the VTA is the dopamine neuron, which provides major projections to the NAc and the PFC. DA neurons exhibit two patterns of firing activity: slow frequency (tonic) single-spike firing and rapid (phasic) burst firing. In response to food or drugs of abuse, burst firing generates a large increase in DA release in VTA target areas including the NAc, which is believed to represent a mechanism through which VTA DA neurons encode reward-related signals (Grace, Floresco, Goto, & Lodge, 2007). Afferent inputs to the VTA can selectively alter the activity states of DA neurons. Whereas excitatory inputs from the PFC as well as the lateral hypothalamus (Omelchenko & Sesack, 2007) can transition DA cells to burst firing, inhibitory inputs from the ventral palladium mediate tonic activity (Floresco, West, Ash, Moore, & Grace, 2003).

As much as 35% of VTA neurons are GABAergic and do not contain tyrosine hydroxylase (TH), a marker for DA neurons (Nair-Roberts et al., 2008). In addition to providing local inhibition, these GABA neurons also project to the NAc and PFC (Carr & Sesack, 2000a; Carr & Sesack, 2000b). While only 2-3% of VTA neurons are glutamatergic, these cells make up highly enriched subpopulations in the medial/rostral VTA (Hur & Zaborszky, 2005). A large proportion of this glutamatergic subpopulation lacks TH

expression (Yamaguchi, Sheen, & Morales, 2007) and is thought to affect both dopaminergic and GABAergic transmission within the VTA (Dobi, Margolis, Wang, Harvey, & Morales, 2010). A minority of glutamatergic subpopulation neurons in the VTA express both VgluT2 and TH, co-release DA and glutamate, and project to the NAc and PFC (Stuber, Hnasko, Britt, Edwards, & Bonci, 2010; Yamaguchi et al., 2007). Although it is known that glutamate in these dopaminergic cells is important for sequestering DA into pre-synaptic vesicles and evoked DA release specifically in the ventral striatum (Hnasko et al., 2010), the postsynaptic function of glutamate release from VTA dopaminergic neurons remains unclear.

BDNF infusion into the VTA has been demonstrated to promote weight loss through unknown mechanisms (Berhow et al., 1995). The VTA is itself a major source of BDNF (Seroogy et al., 1994) from where it can be released locally and act on local TrkB receptors to enhance dopamine neuron excitability in the VTA (L. Pu, Liu, & Poo, 2006). BDNF can additionally be anterogradely transported from the VTA to the NAc (Altar et al., 1997).

Prefrontal Cortex

The PFC also plays a critical role in the valuation of reward within the mesolimbic system. Pyramidal neurons receive dopaminergic innervations from the VTA. They also project to and form glutamatergic synapses with both the NAc and VTA (Kalivas & Volkow, 2005). These excitatory projections from the PFC play an important role in regulating the activity of VTA neurons and ascending DA projections. For example, strong motivational stimuli which activate glutamatergic neurons in the PFC stimulate burst firing in VTA DA neurons and increase extracellular DA in the NAc (Murase, Grenhoff, Chouvet, Gonon, &

Svensson, 1993). Inactivation of the PFC produces the opposite response (Murase et al., 1993). Since glutamatergic inputs from the PFC do not synapse onto VTA DA neurons targeting the NAc, this regulation is likely to take place indirectly (Carr & Sesack, 2000a). Nevertheless, this connectivity is considered behaviorally relevant, particularly since dysfunction in the PFC has been linked to addiction behaviors including binge eating (Goldstein & Volkow, 2011).

The PFC comprises another major source of BDNF in the mesolimbic system, from where it can be transported via glutamatergic afferents to the VTA or to GABAergic medium spiny neurons within the NAc, a region of minimal BDNF expression (Altar et al., 1997; Conner et al., 1997). TrkB is expressed in the PFC (Numan & Seroogy, 1999; Yan et al., 1997) suggesting that glutamatergic neurons are also responsive to BDNF.

Nucleus Accumbens

The NAc makes up the ventral striatum, a key region mediating the reward and motivation to consume palatable food. Approximately 95% of the NAc consists of inhibitory, medium-spiny neurons which receive strong dopaminergic input from the VTA, glutamatergic input from the PFC, and also send GABA-ergic projections toward the VTA. In response to food, sucrose or drugs of abuse, DA neuron burst firing in the VTA generates a large increase in dopamine release in the NAc (Bassareo & Di Chiara, 1997; Ghiglieri et al., 1997; Rada, Avena, & Hoebel, 2005).

The NAc is further divided into two anatomical and functionally distinct subregions; the Nac Shell, and NAc Core. Dopamine signaling in the NAc shell, which comprises a transitional zone between the striatum and the limbic system, is thought to encode responses to rewarding stimuli. In support of this, lesions to the NAc shell (but not the core), impair VTA stimulation-induced feeding (Trojniar, Plucinska, Ignatowska-Jankowska, & Jankowski, 2007). Furthermore, inhibition of neurons in the NAc shell by administration of excitatory amino acid antagonists or GABA agonists elicits intense feeding in satiated rats (Baldo, Alsene, Negron, & Kelley, 2005; Stratford, Swanson, & Kelley, 1998). The NAc core, which immediately surrounds the anterior commissure, is considered a functional extension of the dorsal striatum and basal ganglia and seems to be particularly important for initiating the locomotor effort necessary to obtain reward (Szczypka et al., 2001). Notably, intake of palatable food induces dopamine release preferentially in the NAc shell compared to the core (Tanda & Di Chiara, 1998). Furthermore, *in vivo* microdialysis studies in rats reveals that whereas unpredicted consumption of palatable food releases dopamine in the shell, food anticipation is related to secretion in the core (Bassareo & Di Chiara, 1999).

BDNF protein in the NAc is primarily VTA- and PFC-derived, from where it is anterogradely transported (Altar et al., 1997). Recent evidence suggests that minimal levels of BDNF may also be produced locally in NAc neurons upon cocaine self-administration and that this may even be necessary in the persistence of dopamine-dependent behaviors (Graham et al., 2007).

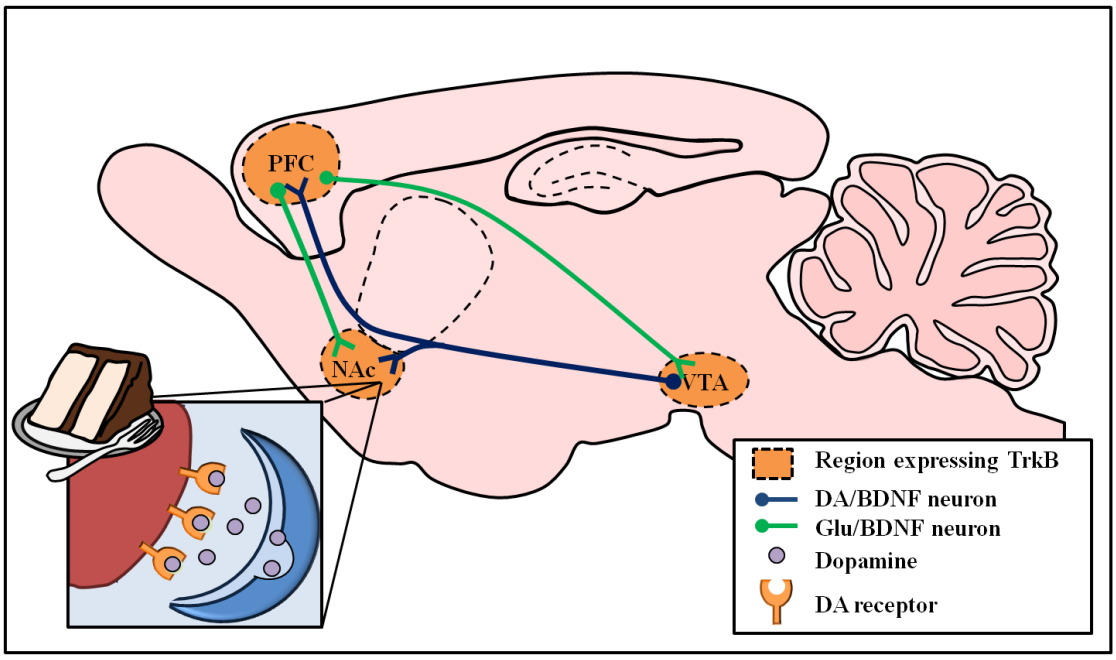

Figure 1.4

BDNF signaling in the mesolimbic dopamine system. Dopamine (DA) neurons originating in the ventral tegmental area (VTA) project to the prefrontal cortex (PFC) and nucleus accumbens (NAc), where they release DA in response to palatable food intake (Inset). BDNF is expressed in both the VTA and the PFC from where it can be transported to the nucleus accumbens (NAc) via DA and glutamatergic (Glu) afferents, respectively. BDNF released from these neurons may act on TrkB receptors expressed on neurons in the NAc, PFC, and VTA to modulate this circuitry and influence the control of hedonic feeding.

Dopaminergic Control of Food Intake

Mounting evidence in rodents and humans implicate DA signaling in the reinforcing behavioral effects of palatable food and obesity (Thanos, Michaelides, Piyis, Wang, & Volkow, 2008). In support of this, evoked DA release is severely attenuated in the NAc of diet-induced obese and obesity-prone rats which also display decreased DA and D2R levels (Geiger et al., 2008; Geiger et al., 2009; Pothos, Sulzer, & Hoebel, 1998). Obese rats with leptin impairment (Zucker rats) also display significant deficits of D2R in the NAc (Thanos et al., 2008). Similarly, hyperphagic and obese leptin deficient *(ob/ob)* mice display decreased evoked DA release (Hommel et al., 2006) and decreased extracellular levels of DA in the NAc (Fulton et al., 2006). The cumulative data suggest that decreased DA signaling in the NAc promotes hedonic feeding. This is compatible with the finding that administration of DA agonists to *ob/ob* mice normalizes hyperphagia and body weight (Bina & Cincotta, 2000). Recent evidence demonstrating significant DA impairment in neo-natal (pre-obese), obesity prone rats further supports the possibility that altered dopaminergic transmission is involved the development of, rather than a consequence of, obesity in rodents (Geiger et al., 2008).

Limited evidence for dopaminergic dysfunction in obesity extends to humans as well. Striatal D2R levels are decreased in obese individuals relative to their BMI (G. J. Wang et al., 2001). Furthermore, individuals with a TaqIA restriction fragment length polymorphism associated with decreased D2R availability in the dorsal striatum are also more likely to be obese than those without the allele (Stice, Spoor, Bohon, & Small, 2008). Interestingly, the amount of striatal DA release in humans correlates with the degree of pleasure obtained from

eating palatable food (D. M. Small, Jones-Gotman, & Dagher, 2003). Altogether, these data offer evidence supporting the notion that alterations in dopaminergic transmission are linked to the development of obesity.

BDNF Influences Dopamine Signaling

BDNF signaling has well documented effects on dopamine signaling and DA-dependent behaviors in the mesolimbic dopamine system. BDNF application acutely potentiates the release of DA from rat striatal slices and primary mesencephalic neurons (Blochl & Sirrenberg, 1996; Goggi, Pullar, Carney, & Bradford, 2002). Notably, this requires the activation of TrkB through PI3-K and MAPK signaling pathways, but not PLC-γ (Goggi, Pullar, Carney, & Bradford, 2003). BDNF from dopamine neurons is also required for the normal expression of dopamine D3 receptor, a D2- like DA receptor expressed primarily in the VTA and NAc (Guillin et al., 2001).

BDNF and TrkB have also been implicated in drug-induced plasticity in the mesolimbic dopamine system. For example, intra-accumbens injections of BDNF or TrkB antibody, suppresses methamphetamine induced dopamine release and induction of dopamine related locomotor behaviors (Narita, Aoki, Takagi, Yajima, & Suzuki, 2003). Transient increases in BDNF in the NAc shell (arising from local synthesis) during cocaine self-administration promote increases in drug-taking and drug-seeking behavior (Graham et al., 2007). Localized BDNF knockdown in either VTA or NAc neurons reduces cocaine place conditioning. Moreover, TrkB knockdown in the NAc but not the VTA reduces cocaine place conditioning, indicating that BDNF signaling through post-synaptic TrkB

receptors on medium spiny NAc neurons is important in drug reward behavior (Graham et al., 2009). BDNF is also necessary to potentiate excitatory transmission onto neurons in the VTA during cocaine withdrawal (L. Pu et al., 2006).

The cumulative evidence indicates that the role for BDNF in the modulation of DA transmission in the mesolimbic dopamine system is behaviorally relevant. Moreover, because DA has known and robust effects on food intake, and since BDNF administration in the VTA induces weight loss (Berhow et al., 1995), it is altogether possible that neurotrophin action in this pathway modulates hedonic feeding. In this thesis, I describe work testing this hypothesis.

Contributions of this Thesis

The Actions of BDNF in Energy Homeostasis

Recent research has advanced our understanding of biological risk factors contributing to obesity, including perturbed BDNF signaling. Rodent and human studies attest to the critical roles of BDNF and its receptor, TrkB, in the neural circuitry regulating energy homeostasis. However, mechanisms underlying the satiety effects of BDNF in the brain and its role in the pathogenesis of obesity are not completely understood.

To gain insight into these mechanisms, in Chapter 2 of this thesis, we examined the trancriptome of cells from the VMH in wild type and $BDNF^{2L/2LCk\text{-}cre}$ mice. Our lab had demonstrated previously that BDNF in this region was required for appetite control. Among the differentially regulated genes in mutant mice, our analysis revealed a significant deficit in

the gene encoding $\alpha_2\delta$-1, a subunit of high voltage-gated calcium channels and thrombospondin receptor. We used a pharmacological approach to study the role of α2δ-1 in food intake and body weight by chronically inhibiting α2δ-1 via gabapentin infusion into wild type VMH. We also examined whether reduced α2δ-1 triggered the hyperphagia and obesity in $BDNF^{2L/2LCk\text{-}cre}$ mice. To do this, I virally delivered $\alpha_2\delta$-1 to selectively rescue expression in the VMH of BDNF mutants and explored the effects on food intake, body weight. I also examined serum levels of glucose, insulin, and leptin in these animals, which are elevated as a result of obesity and serve as key indicators of metabolic dysfunction. Finally, to gain insight into the mechanisms through which α2δ-1 might mediate satiety, we examined calcium channel currents in VMH neurons from WT and $BDNF^{2L/2LCk\text{-}cre}$ mice. I also used quantitative RT-PCR immunohistochemical techniques to explore whether the satiety effects of α2δ-1 were mediated by interactions with TSPs.

Our findings provide a mechanistic understanding of how BDNF signaling facilitates homeostatic appetite control and offer insight on new strategies for the treatment and prevention of obesity. Moreover, they identify a previously unrecognized role for α2δ-1 in food intake and body weight regulation. A comprehensive discussion of our results and future directions are discussed in Chapter 4.

The Actions of BDNF in Hedonic Food Intake

The mesolimbic dopamine system has long been recognized as the neural circuitry orchestrating the dopamine-dependent behavioral effects to drugs of abuse and natural

rewards such as food, albeit through poorly defined mechanisms. Recent evidence suggests BDNF signaling through its receptor, TrkB, may modulate behavioral relevant plasticity in the mesolimbic dopamine system. In Chapter 3 of this thesis, we sought to ascertain whether alterations in BDNF signaling within this circuitry may also underlie abnormal food intake behavior leading to obesity in $BDNF^{2L/2LCk\text{-}cre}$ mice.

To this end, we utilized a multidisciplinary approach to explore the role of BDNF in palatable food consumption and potential alterations in DA signaling in mice with central depletion of BDNF. We used amperometric recordings in brain slices from WT and $BDNF^{2L/2LCk\text{-}cre}$ mice to study the role of BDNF on evoked release of dopamine in the NAc, a synaptic target of the VTA in the mesolimbic dopamine system. Using pharmacological approaches I explored the effects of DA signaling *in vivo* in $BDNF^{2L/2LCk\text{-}cre}$ mice. For this, I administered a D1R agonist and studied the effects on food intake in mice with or without the central depletion of BDNF in the brain. Finally, to identify essential sources of BDNF within the mesolimbic pathway, I used viral-mediated delivery of Cre-recombinase to selectively deplete *Bdnf* in the VTA of mature, floxed-BDNF mice and studied the effects of food intake and body weight.

Our findings here significantly contribute to the understanding of the multiple mechanisms through which BDNF regulate feeding behavior. These data moreover help to define neural substrate-specific roles for BDNF wherein dysfunction might contribute to the pathogenesis of eating disorders and obesity. A full discussion of these results and their impact on future directions of research are included in Chapter 4.

Chapter 2:

Hypothalamic dysfunction of the calcium channel subunit and thrombospondin receptor α2δ-1 underlies the overeating and obesity triggered by BDNF deficiency

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Abstract

Brain-derived neurotrophic factor (BDNF) and its receptor, TrkB, are critical components of neural feeding circuits. Diminished BDNF signaling is associated with hyperphagia and severe obesity in humans and rodents. However, the underlying pathological mechanisms are poorly defined. Here we show that reduced cell surface expression of the calcium channel subunit and gabapentin/thrombospondin receptor, α2δ-1, in the ventromedial hypothalamus (VMH) triggers excessive feeding in BDNF deficient mice. Furthermore, α2δ-1 inhibition by gabapentin infusion into wild type VMH increases food intake and body weight. Importantly, viral-mediated α2δ-1 rescue in BDNF mutant VMH attenuates overeating and body weight gain and improves metabolic dysfunction. Normal calcium currents in BDNF mutants indicate calcium channel-independent effects of α2δ-1 and implicate α2δ-1-thrombospondin interactions, which facilitate synapse assembly. Our findings identify a novel role for α2δ-1 in appetite control modulated by BDNF and suggest a mechanism underlying the weight gain in humans treated with gabapentinoid drugs.

Introduction

BDNF and its receptor, TrkB, promote neuronal survival, differentiation and synaptic plasticity (Patapoutian & Reichardt, 2001). They are also critical components of the neural circuitry controlling food intake and body weight. Accordingly, $BDNF^{2L/2LCk\text{-}cre}$ mice with central depletion of BDNF exhibit excessive feeding and severe obesity (Rios et al., 2001).

In humans, BDNF haploinsufficiency and the *Bdnf* Val66met polymorphism, which is carried by 27% of the U.S. population, have been linked to elevated food intake and obesity (Shimizu, Hashimoto, & Iyo, 2004). While the prevalence of obesity and its associated medical complications continue to rise globally, the mechanisms underlying the satiety effects of BDNF and its role in the pathogenesis of obesity are poorly understood.

The cumulative data indicate that the ventromedial hypothalamus (VMH), a critical region for the regulation of homeostatic feeding, is an important site of BDNF/TrkB action for the regulation of appetite. For example, energy status robustly influences expression of BDNF and TrkB in the VMH (Tran, Akana, Malkovska, Dallman, Parada, & Ingraham, 2006a; Unger et al., 2007; Xu et al., 2003) and delivery of exogenous BDNF to this region reduces feeding and weight gain (C. Wang, Bomberg, Levine et al., 2007). Moreover, we showed that selective *Bdnf* deletion in the VMH of adult mice elicits hyperphagia and obesity (Unger et al., 2007). Here, we describe multidisciplinary studies examining the mechanisms underlying the satiety effects of BDNF in the VMH. These findings help to define the mechanistic action of BDNF/TrkB signaling in appetite control and offer insight on new strategies for the treatment and prevention of obesity.

Methods:

Animals: Male $BDNF^{2L/2LCk\text{-}cre}$ were generated as previously described (Rios et al., 2001). Briefly, for the generation of mice with floxed *Bdnf* alleles, loxP sites were inserted around the single coding exon of *Bdnf*. BDNF conditional mutants were generated by crossing mice carrying floxed *Bdnf* alleles with transgenic mice in which expression of cre recombinase was driven by the α-calcium/calmodulin protein kinase II (CamKII). The

mutant mice are depleted of BDNF in excitatory neurons throughout the brain except the cerebellum. Age-matched littermate wild type controls were used in every experiment to reduce genetic background differences on the C57BL/6 129 strain. All of the procedures were approved by the Institutional Animal Care and Use Committee at Tufts University and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Laser Capture Microdissection (LCM): Wild type and $BDNF^{2L/2LCk\text{-}cre}$ mice were sacrificed and their brains were rapidly extracted and frozen. 10 μm-thick sections representing two independent coronal levels of the VMH were sliced on a Leica CM1900 Cryostat. Sections were subsequently hydrated in a series of ethanols, stained in cresyl violet, and dehydrated in ethanol, histoclear and by vacuum desiccation. Unilateral (for Genechip) or bilateral (for Quantitative RT-PCR) laser capture microdissection (LCM) of cresyl-violet stained cells in the VMH was performed using the Arcturus Pixcell Iie LCM system. Total RNA was extracted from captured cells using the Picopure RNA extraction kit (Arcturus, Sunnyvale, CA).

***GeneChip Analysis*:** Target cRNAs from each experimental animal were fragmented and independently hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetryx, Santa Clara, CA). GeneChips were then washed and stained with Streptavidin R-phycoerythrin (Molecular Probes, Carlsbad, CA) and following washes, scanned using the GeneChip scanner. Data analysis was conducted using the Bioconductor suite of programs and the RankProd algorithm was used to calculate the list of differentially expressed genes. RankProd also incorporates a non-parametric permutation test to calculate the associated p-value and false discovery rate (FDR) for the significance of detection that allowed for a 0.05

cut-off for the list of differentially expressed genes at a significant FDR. Genes that were changed in both the comparison of wild type and $BDNF^{2L/2LCk\text{-}cre}$ mice as well as the comparison of AAV2/1-GFP-treated and AAV2/1-Cre-treated mice were further analyzed.

In situ *hybridization*: Twelve μm-thick tissue sections containing the VMH were hybridized for 16 h at 60°C with a ^{35}S-labeled, antisense riboprobe representing bases 2473-3135 of the $\alpha_2\delta$-1 cDNA. Specificity of this riboprobe was confirmed by lack of hybridization to tissue with the sense riboprobe. After the hybridization step, sections were stringently washed and place on x-ray film overnight.

***Immunofluorescence*:** Wild type mice (male, 8-10 weeks old) were anesthetized with isoflurane and transcardially perfused with 10 ml of cold saline followed by 30 ml of 4% paraformaldehyde (PFA), pH 7.2. Brains were immediately removed, post-fixed in 4% PFA for 2 hours at room temperature, cryoprotected in a 30% sucrose solution, and frozen in mounting media until 25μm-thick coronal sections representing the whole rostral-caudal extent of the VMH were obtained using a Leica CM1900 Cryostat. For α2δ-1/TrkB immunolabeling, sections were rinsed in 1X PBS and blocked in 5% normal donkey serum/0.1% Triton X-100 in 1X PBS for 90 minutes at room temperature (RT). Sections were incubated for 16 hours at 4°C with mouse anti- α2δ-1 (1:100, Sigma) and rabbit anti-TrkB (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). For TSP-3, sections were blocked in 10% normal donkey serum/0.1%TritonX-100 in 1xPBS for 1 hour at RT and subsequently incubated in mouse anti-TSP3 (1:50, A-12 Santa Cruz) for 48 hrs at 4°C. For α2δ-1/TSP-3 immunolabeling, sections were blocked in 10% normal donkey serum in 1xPBS before incubation with rabbit anti-α2δ-1 (1:50, Alomone Labs, Israel) and mouse anti-TSP3

(1:50, A-12 Santa Cruz) in the absence of TritonX-100. Following primary incubation, sections were rinsed in 1xPBS and incubated with corresponding donkey anti-rabbit Cy3 and/or donkey anti-mouse FITC conjugated secondary antibodies (1:200, Jackson ImmunoResearch, Westgrove, PA) for 1 hour at RT. Tissue sections were finally mounted on Superfrost plus slides and coversliped using Vectashield (Vector Labs, Burlingame, CA).

Delivery of Gabapentin and Saline to the VMH: The VMH of WT male mice (8-10 weeks of age was infused with Gabapentin or saline via cannulas (Plastics One, Roanoke, VA) implanted unilaterally using the coordinates: anteroposterior – 1.5 mm; mediolateral, + 0.5 mm; dorsoventral, - 5.7 mm from Bregma. Cannulas were connected to Alzet Model 1004 Osmotic minipumps (Alzet, Cupertino, CA) containing either 100 μl of either GBP (150 μg/μl; Sigma) or sterile saline. Mice were individually housed and allowed 3 days to recover before daily food and body weight monitoring began for the subsequent 7 days.

Stereotaxic of AAV-α2δ-1 and AAV–GFP to the VMH: AAV1 vectors were produced, purified, and titered as previously described (Broekman, Comer, Hyman, & Sena-Esteves, 2006). Mouse α2δ-1b cDNA (a gift from T. Angelotti) was cloned into the AAV1 vector plasmid. Adeno-associated virus (0.75 μl bilaterally; 5.2 x 10^{12} infectious units/ml) encoding α2δ-1 (AAV-α2δ-1) or GFP (AAV-GFP) was delivered to the VMH of anesthetized $BDNF^{2L/2LCk\text{-}cre}$ or wild type mice (male, 7-9 weeks of age) using the following coordinates: anteroposterior – 1.5 mm; mediolateral, +/- 0.5 mm; dorsoventral, - 5.4 mm. Virus was administered bilaterally using a 10 μl Hamilton syringe with a 33-gauge needle attached to a digital stereotaxic apparatus and an infusion microsyringe nanopump (KD Scientific, Holliston, MA) at a rate of 0.1 μl/min. The needle was held in place for 10 min

after injection to permit diffusion of the virus and to minimize backflow after needle retraction. Mice were given 1 week to recover before weekly food intake and body weight monitoring began.

Food Intake and Body weight Measurements: Mice were individually housed with unrestricted access to standard diet (Tekland global diet). Premeasured amounts of food were given such that caloric intake could be calculated based on grams of food consumed (3.3 Kcal/g). Food intake and body weights of male mice receiving GBP or saline were measured daily for 1 week. For AAV mice, food intake was measured once per week for 18 weeks. The changes in food and body weight were measured at the same time of day each week.

Insulin, Leptin, & Glucose Measurements: To measure levels of insulin and leptin, mice were fasted for 16 hrs, anesthetized, and prepared for retro-orbital bleed. 300 μL of blood was collected using hematocrit glass capillaries and EDTA (1.75 mg/mL final) as an anticoagulant. Samples were promptly centrifuged at 2,000 x g for 15 min at 4C. Plasma was transferred to clean tubes and used for measurement in Insulin (Millipore) and Leptin (Phoenix Pharmaceuticals, Inc.) Immunoassay kits per manufacturer's instructions. Blood glucose concentrations were measured from tail bleeds using a Freestyle Blood Glucose Monitoring System (Abbot Diabetes Care Inc.; Alameda, CA). For the Glucose Tolerance Test, 2g/Kg D-Glucose was administered I.P following a baseline (0) measurement. Bloods samples were then collected to measure circulating blood glucose at 15, 30, 60, 120 min post-injection.

Locomotor Activity: Mice were housed individually in standard 15×24 cm plastic cages on a 12-h reverse light/dark cycle. Motor activity was monitored using the Smart Frame Activity System consisting of photobeam frames that surrounded the animal's home cage. Locomotor readouts (beam breaks) were subsequently recorded for 3 undisturbed days using MotorMonitor software (Hamilton/Kinder).

Quantitative RT-PCR: RNA was extracted from VMH, ARC, and DMH tissue samples dissected by laser capture, as described. Reverse transcription to generate cDNA was conducted with 75 ng of RNA and using 200 units of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and 150 ng of random primers (Invitrogen, Carlsbad, CA) in a 20 μl reaction. Real time PCR amplification was performed using a MX-3000P Stratagene cycler and 2x SYBR green PCR master mix (Qiagen, Valencia, CA). For each set of primers, the specificity of the product amplification was confirmed by dissociation curve analysis and agarose gel electrophoresis. Furthermore, curves were created using serial dilutions and the efficiencies for each primer set was calculated. The amplification efficiency for all the primers used in this study was >90 %. For each target primer set, a validation experiment was performed to demonstrate that the PCR efficiencies were approximately equal to those of the reference gene, cyclophilin. A two-step protocol was used: 95°C for 10 min and 45 cycles with 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The following intron-spanning primers were used:

α2δ-1:	(F) 5'CAAGCGGAACAGACTTCTGATGGT3',
	(R) 5'AGTAGGTAGTGTCTGCTGCCAGAT3';
BDNF (exon-1):	(F) 5′CCTGCATCTGTTGGGGAGAC3′;
	(R) 5′GCCTTGTCCGTGGACGTTTA3′;

TSP-1:	(F) 5’ATGGGGTTGGAGATCAGTGT 3’,
	(R) 5’GTCATCGTCATGGTCACAGG 3’;
TSP-2:	(F) 5’TGACGGAGACGATGTTTTCA3’,
	(R) 5’GGGCAGTTGTCTTGGTTGTT 3’;
TSP-3:	(F) 5’ATGGAGAAGCCGGAACTTTGG 3’,
	(R) 5’AGTGAGTAAAGCTGTCCGAATCT 3’;
Cyclophilin:	(F) 5’CACCGTGTTCTTCGACATCA3’,
	(R) 5’CAGTGCTCAGAGCTCGAAAG3’

All samples were analyzed in triplicates, and non-template controls were included to ascertain any level of contamination. Data obtained were analyzed using the comparative Ct method. Every experiment was repeated at least once.

Cell-Surface Biotinylation: Mice (male, aged 8-11 wks) were anesthetized with Isoflurane, decapitated and their brains rapidly removed into ice-cold artificial cerebrospinal fluid (aCSF) composed of the following: 130mM NaCl, 3.5 mM KCl, 1.10 mM KH_2PO_4, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 30 mM $NaHCO_3$, and 10 mM Glucose. aCSF was gassed with 95%-5% oxygen/carbon dioxide mixture. Coronal slices (350 μm thick) encompassing the VMH were prepared on a VT 1000S Vibrostome (Leica Microsystems, Heidelberg, Germany) and allowed to recover in oxygenated aCSF for 90 min at 32°C. Following recovery, slices were incubated in 1 mg/mL Sulfo-NHS-SS-Biotin (Peirce Thermo Scientific, Rockford, IL) in aCSF for 30 min on ice. Excess Biotin was quenched by incubating slices in 50mM Tris-HCl (pH 8.0) followed by rinses in 1xPBS. The VMH was bilaterally dissected from 2 animals (totaling 4 slices) and pooled to compose 1 sample. Samples were snap frozen and sonicated in lysis buffer composed of: 20 mM Tris-HCl (pH 8.0), 150 mM

NaCl, 5mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 1% v/v TritonX-100, 0.1% SDS, and Halt Protease Inhibitor Cocktail (Pierce ThermoScientific). Upon centrifugation at 16,000 x g to remove cellular debris, supernatent was collected and then subjected to a BCA Protein Assay (Peirce ThermoScientific). 50 μL of (total) protein were loaded with 50 μL NeutrAvidin beads (1:1 slurry, Peirce ThermoScientific) to immunoprecipitate the biotinylated protein. Samples were allowed to conjugate overnight at 4°C with constant agitation. Beads were precipitated by centrifugation 500 x g and the supernatant collected as the unbiotinylated (cytosolic) protein fraction. Beads were washed twice with both cold lysis buffer and cold lysis buffer supplemented with 500 mM NaCl. After a final wash in cold lysis buffer, beads were re-suspended in 20 μL of sample buffer containing 100 mM DTT. The biotinylated (cell surface) protein fraction was eluted by boiling 10 min in the reducing sample buffer.

Western Blot: Protein samples were separated on a 7.5% SDS-PAGE gel and transferred to Immun-blot PVDF membranes (Bio Rad, Hercules, CA). The membranes were subsequently blocked with non-fat milk 5% w/v in TBS-Tween pH 7.4 for 1 hour at room temperature and probed with primary antibody in blocking solution overnight at 4°C. Membranes were rinsed with TBS-Tween, incubated with peroxidase-conjugated secondary antibodies at room temperature for 1 hour, and rinsed again before final reaction in ECL chemoluminescent detection solution (GE Healthcare, Amersham, UK) for 5 minutes. Images were acquired using a Fuji film LAS-4000 Image reader. Densitometry of blots was performed using Quantity One analysis software (BioRad, Hercules, CA) and normalized to β-tubulin or 10% total protein,. The following antibodies were used: rabbit anti-Ca_V α2δ-1 (1:1,000, Alomone Labs, Jerusalem, Israel), mouse anti-β tubulin (1:40,000 Sigma, St. Louis,

MO), goat anti-rabbit IgG-HRP (1:5,000 Santa-Cruz Biotechnology, Santa-Cruz, CA), goat anti-mouse IgG -HRP (1:5,000 Jackson Immunoresearch, West Grove, PA).

Electrophysiology: Mice were anesthetized, decapitated and brains rapidly removed. Briefly, a block of tissue containing the hypothalamus was sliced in the coronal plane (250 μm) in ice-cold high-sucrose artificial cerebrospinal fluid (aCSF: in mM): 250 sucrose, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, 2.5 $MgCl_2$. Slices were equilibrated in a holding chamber prior to recording. For recordings, slices were placed in submersion-type recording chamber continuously superfused with oxygenated ACSF at 32°C. For voltage clamp recording of I_{Ca}, the aCSF solution contained (in mM): 120 NaCl, 20 KCl, 10 glucose, 26 $NaHCO_3$, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 5 4-aminopyridine (4AP), 5 CsCl, 10 tetraethylammonium (TEA)-Cl, and 0.0005 TTX, pH 7.3, 290 mOsm (gassed with 95% O_2 and 5% CO_2); the pipette solution contained the following (in mM): 120 Cs-gluconate, 10 HEPES, 10 EGTA, 0.5 $CaCl_2$, 4 Mg-ATP, 0.4 NaGTP, and 20 TEA-Cl, pH 7.3 (titrated with CsOH, 310 mOsm). Bicuculline (10 μM), APV [D-(L)-2-amino-5-phosphonovaleric acid] (50 μM), and NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide) (10 μM) were included in the bath solution to block ionotropic GABAergic and glutamatergic currents. Electrodes (2.5 - 4 MΩ) contained (in mM): 120 Cs-gluconate, 10 HEPES, 10 EGTA, 0.5 $CaCl_2$, 4 Mg-ATP, 0.4 NaGTP, and 20 TEA-Cl, pH 7.3 (titrated with CsOH, 310 mOsm). VMH neurons were visualized with an upright microscope (Nikon Eclipse FN-1) using infrared and differential interference contrast illumination. Whole-cell voltage-clamp recordings were made with a multi-clamp-700B amplifier (Molecular Devices). Neurons were held at -70 mV and currents were elicited by a 250 ms depolarizing test pulse from -70 to + 50 mV at 10 mV increments. All currents were

corrected for leak and capacitive currents by a P/-4 protocol. Access resistance (*R*a), series resistance (*R*s), and membrane capacitance (*C*m) were continually monitored. Amplitude of Ca^{2+} current, time to peak and inactivation of the maximal current traces (at -20mV or -10 mV) at the end of the 250 ms depolarization (R250) were analyzed in pClamp10. Current density was calculated by divind the peak current amplitude by Cm (pA/pF).

Statistical Analysis: All values are expressed as mean +/- S.E.M. Statistical comparisons were performed using Graphpad Prism (ANOVA) and Microsoft Excel (TTEST) as dictated by the data. Students unpaired TTESTs were performed to analyze transcript, protein, food intake and bodyweight, blood serum levels, mean current amplitude, time to activation, and R250. For the analysis of food intake and body weight over time as well as the glucose tolerance test, a two-way ANOVA with repeated measures was performed with time and treatment as variables for comparison. Variables were considered statistically significant when $p < 0.05$.

Results

Cell surface expression of α2δ-1 is reduced in the VMH of BDNF$^{2L/2LCk\text{-}cre}$ mice

To gain further insight into the molecular mechanisms underlying the anorexigenic effects of BDNF, we conducted an analysis of the transcriptome of cells laser-captured from the VMH of wild type (WT) and BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant mice. A total of 64 differentially expressed genes with a false discovery rate of < 0.05 were identified, of which 61 genes were downregulated and 3 upregulated (Table 2.1). We focused our investigations

on *CACNA2D1*, which was down regulated 4.7 fold in the VMH of BDNF$^{2L/2LCk\text{-}cre}$ mice. Of note, this deficit was also evident in mice in which *Bdnf* was selectively deleted in the adult VMH (data not shown), indicating that this was not a developmental defect and that BDNF synthesized in the VMH acts locally to affect *CACNA2D1*.

CACNA2D1 encodes the α2δ-1 subunit of high voltage-gated calcium channels (Ca_V). α2δ-1 functions to facilitate trafficking of Ca_V to the cell surface and thereby increase calcium current density and neurotransmitter release (Bauer, Tran-Van-Minh, Kadurin, & Dolphin, 2010; A. Davies et al., 2007). It also promotes excitatory synaptogenesis in a calcium channel-independent manner by acting as a thrombospondin receptor (Eroglu et al., 2009). α2δ-1 is enriched in the VMH (Fig. 2.1A) (Kurrasch et al., 2007) and in other appetite regulating areas of the brain (Cole et al., 2005; Taylor & Garrido, 2008) suggesting a role in energy homeostasis. Furthermore, systemic administration of the anti-epileptic and anti-nociceptive drugs gabapentin and pregabalin, which selectively inhibit α2δ-1 (Gee et al., 1996), was associated with body weight gain in patients, albeit through unknown mechanisms (DeToledo et al., 1997; Hoppe et al., 2008). We examined whether reduced α2δ-1 activity underlies the hyperphagic behavior and obesity in BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant mice.

We first confirmed the α2δ-1 deficit by analyzing protein levels. Surprisingly, total protein levels of α2δ-1 were normal in the VMH of BDNF$^{2L/2LCk\text{-}cre}$ mice. However, cell-surface expression was reduced by 36% (p = 0.002) (Fig. 2.1B). A concomitant 58% increase in cytosolic α2δ-1 protein was also evident in mutant VMH cells compared to WT controls (p = 0.02) (Fig. 2.1B), indicating intracellular retention of α2δ-1. Together, the data indicate that BDNF is required for normal cell surface expression of α2δ-1 in the VMH.

Accordingly, confocal analysis of WT brain sections co-immunolabeled with anti-TrkB and anti-α2δ-1 revealed high levels of co-localization of these molecules in cells in the VMH (Fig. 2.1C), indicating that cells expressing α2δ-1 are sensitive to BDNF.

Inhibiting α2δ-1 in the wild type VMH induces hyperphagia and weight gain

We directly interrogated the role of α2δ-1 in appetite control by pharmacologically inhibiting its function in the VMH. Gabapentin (GP), which is transported inside the cell via the L α-amino acid transporter and acts primarily at intracellular locations to inhibit cell surface expression of α2δ-1 (Bauer et al., 2009; Hendrich et al., 2008), was chronically infused into the VMH of wild type mice. GP-treated WT mice ate 39% more standard chow compared to WT mice infused with saline ($p = 0.02$) (Fig. 2.2A). Additionally, GP treatment induced a significant increase in percentage body weight gained over 7 days of treatment compared to saline infusion ($p = 0.04$) (Fig. 2.2B). These data identified α2δ-1 as a novel regulator of appetite acting in the VMH.

Viral delivery of α2δ-1 to the VMH of BDNF$^{2L/2LCk\text{-}cre}$ mice attenuates their hyperphagia, weight gain, and metabolic syndrome

Next, we examined the effect of rescuing α2δ-1 in the BDNF mutant VMH. For this, we delivered adeno-associated viral vectors encoding α2δ-1 (AAV-α2δ-1) or green fluorescent protein (AAV-GFP) bilaterally to the VMH of BDNF$^{2L/2LCk\text{-}cre}$ mice via stereotaxic surgery. WT mice treated with AAV-GFP (WT-GFP) were included as controls

for normal levels of food intake. Only BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant mice treated with AAV-α2δ-1 (CM-α2δ-1) that exhibited at least 40% increase in α2δ-1 mRNA expression in the VMH were included in the analysis (Fig. 2.2C). At five weeks post-surgery, shortly after AAV-mediated gene expression is expected to peak (Broekman et al., 2006), and continuing until the end of the study, CM-α2δ-1 mice exhibited a 15-20% reduction in food intake relative to CM-GFP mice (Fig. 2.2D). Relative body weight gain of CM-α2δ-1 mice was also significantly reduced compared to CM-GFP animals (Fig. 2.2E). Locomotor activity of CM-α2δ-1 mice was similar to that of CM-GFP mice, suggesting that their reduced body weight gain was not related to increased locomotion (Fig. 2.2F).

BDNF$^{2L/2LCk\text{-}cre}$ mice and mice with selective depletion of BDNF in the VMH exhibit the deleterious metabolic effects of obesity, including hyperleptinemia, hyperinsulinemia, and hyperglycemia (Rios et al., 2001; Unger et al., 2007). We found that the α2δ-1 rescue significantly improved these aspects of the metabolic syndrome. Indeed, CM-α2δ-1 mice displayed a significant reduction in circulating levels of glucose, insulin and leptin compared to CM-GFP mice (Fig. 2.2G). Additionally, whereas CM-GFP mice exhibited a compromised response to a glucose challenge compared to WT-GFP mice, the response of CM- α2δ-1mice was greatly improved and similar to that of WT-GFP controls (Fig. 2.2H and I).

Calcium channel function is normal in BDNF$^{2L/2LCk\text{-}cre}$ mice

To investigate whether the deficit in α2δ-1 in BDNF mutants might affect appetite via a calcium channel-dependent mechanism, we conducted whole-cell recordings in acute brain

slices from WT and BDNF$^{2L/2LCk\text{-}cre}$ mice to measure calcium currents in VMH neurons. Average peak amplitude, voltage dependence, and current kinetics including activation and inactivation were indistinguishable between WT and BDNF$^{2L/2LCk\text{-}cre}$ mice (Fig. 2.3B and C), suggesting that the anorexigenic effects of α2δ-1 in the VMH are unlikely to be related to alterations in calcium-channel function.

Hypothalamic expression of the α2δ-1 ligand TSP-3 is regulated by energy status

A recent report demonstrated that α2δ-1 also induces excitatory synaptogenesis in a calcium channel-independent manner by serving as a receptor for thrombospondins (TSPs) and this effect is inhibited by GP (Eroglu et al., 2009). Because dynamic changes in synaptic connectivity of hypothalamic circuits are thought to contribute to appetite control (Pinto et al., 2004; Sternson et al., 2005), we asked whether the satiety effects of α2δ-1 might be related to interactions with TSPs. We examined the effect of nutritional status on the expression of the α2δ-1 ligands TSP-1, 2 and 3 in cells laser-captured from WT VMH and from energy balance-regulating hypothalamic regions with known synaptic connections with cells of the VMH, including the arcuate nucleus (Arc) and dorsomedial hypothalamus (DMH). Levels of TSP-1 and 2 mRNA were not influenced by energy status in all regions examined (Fig. 2.4). In contrast, TSP-3 mRNA expression was significantly decreased by fasting in the DMH (Fig. 2.5A) but not in the VMH or Arc (Fig. 2.5A). Interestingly, we also discovered that the DMH of BDNF$^{2L/2LCk\text{-}cre}$ mice exhibited a 2-fold elevation in TSP-3 transcripts relative to WT (Fig. 2.5B), suggesting a homeostatic compensatory response to the α2δ-1 dysfunction in the VMH. We also found that α2δ-1 mRNA and protein expression was not affected by metabolic status (Fig. 2.6A and B). These findings suggest that short

term alterations in BDNF content induced by energy status are not sufficient to influence expression of α2δ-1 in the same manner as long term BDNF perturbations, as seen in BDNF mutant mice.

The VMH has reciprocal connections with the DMH (Luiten & Room, 1980; Ter Horst & Luiten, 1987), a hypothalamic region mediating ingestive behavior (Bellinger & Bernardis, 2002). The observed effects of energy status and BDNF depletion on TSP-3 expression in the DMH suggests that TSP-3 might serve as a ligand for α2δ-1 in cells in the VMH, possibly regulating synaptic connectivity between these two hypothalamic nuclei. As a first step to test this concept, we examined the spatial distribution of TSP-3^{+} fibers. We observed TSP-3 immunopositive neuronal fibers traveling between the VMH and DMH (Fig. 2.5C). Furthermore, confocal imaging of non-permeabilized wild type brain sections that were double immunolabeled showed that α2δ-1 receptor clusters overlapped or were in close apposition with TSP-3 stained processes in the wild type VMH (Fig. 2.5D).

Discussion

Here, we show that α2δ-1 is a novel and critical regulator of appetite acting in cells of the VMH and that its function in this context requires BDNF. The satiety effects of α2δ-1 might not be restricted to the VMH as it is also highly expressed in the PVN and DVC (Taylor & Garrido, 2008), energy balance-regulating regions where BDNF signals for satiety (Bariohay et al., 2005; Toriya et al., 2010; C. Wang, Bomberg, Billington, Levine, & Kotz, 2007a). This may explain why food intake in CM-α2δ-1 mice was not completely

normalized to that of WT-GFP controls. Nonetheless, as shown here, direct modulation of α2δ-1 in the VMH is sufficient to alter feeding behavior and bodyweight.

α2δ-1 transcript levels in the BDNF mutant VMH show measured decreases in the transcriptional profiling experiments but not in the RT-PCR analysis. Because α2δ-1 is alternatively spliced (A. Davies et al., 2007), it is possible that the gene array data reflect alterations in RNA splicing in BDNF mutants that ultimately impact cell surface protein expression but not total content of α2δ-1. In support of this idea, introduction of α2δ-1 cDNA using a viral vector, which bypasses this RNA processing step, was capable of attenuating the hyperphagia and metabolic alterations associated with the α2δ-1 deficit in BDNF mutant mice. Future studies will assess how BDNF might influence α2δ-1 transcript processing and how these effects impact α2δ-1 trafficking to the cell membrane.

The finding that calcium currents were not affected in VMH cells of BDNF mutants implicate α2δ-1-thrombospondin interactions, which facilitate excitatory synapse assembly independent of calcium channel function (Eroglu et al., 2009). Because both BDNF and α2δ-1 have demonstrated roles in facilitating synaptogenesis (Aguado et al., 2003; Eroglu et al., 2009; Luikart & Parada, 2006), remodeling of feeding circuits involving cells of the VMH during the fed state is a plausible mechanism. In support of this, rewiring of excitatory synaptic output from the VMH is mediated by nutritional status and satiety signals (Pinto et al., 2004; Sternson et al., 2005). Dynamic changes in synaptic density in the adult VMH induced by the anorexigenic hormone estradiol and estrogen receptor-α were also reported previously (Frankfurt & McEwen, 1991; Sa, Lukoyanova, & Madeira, 2009). Thus, long term alterations in BDNF and concomitant reductions in cell surface α2δ-1, might impact

hypothalamic synaptic connectivity in a manner that reduces the anorexigenic tone and drives obesity.

As shown here, expression of TSP-3, an α2δ-1 ligand that promotes synaptic adhesion and establishment of pre- and post-synaptic specializations (Christopherson et al., 2005), is regulated by energy status. Consistent with a role of TSP-3 in energy balance, TSP-3 null mice exhibit increased body weights at 9 weeks of age, which normalize with age, perhaps due to compensatory effects of other TSP's (Posey et al., 2008). Dynamic regulation of TSP-3 may represent a short-term mechanism mediating synaptic connectivity between hypothalamic nuclei to control food intake. Because α2δ-1 could act pre- or post-synaptically to induce synaptogenesis (Eroglu et al., 2009), future studies will determine whether its interactions with TSP-3 influence excitatory synaptic density onto cells of the VMH and/or the DMH and how these effects are influenced by nutritional cues.

Given the high prevalence of obesity and mutations that interfere with BDNF signaling within the human population (Shimizu, Hashimoto, & Iyo, 2004), defining the mechanistic consequences of BDNF/TrkB signaling is one essential step toward developing novel treatment strategies for obesity and its many associated medical complications. Our investigations provide insight into the pathological processes underlying detrimental increases in body weight induced by perturbed BDNF signaling. Furthermore, they explain the weight gain associated with gabapentinoid drug treatment in patients afflicted with pain and seizure disorders (DeToledo et al., 1997; Hoppe et al., 2008), opening the door for adjunct therapies that minimize this negative side effect.

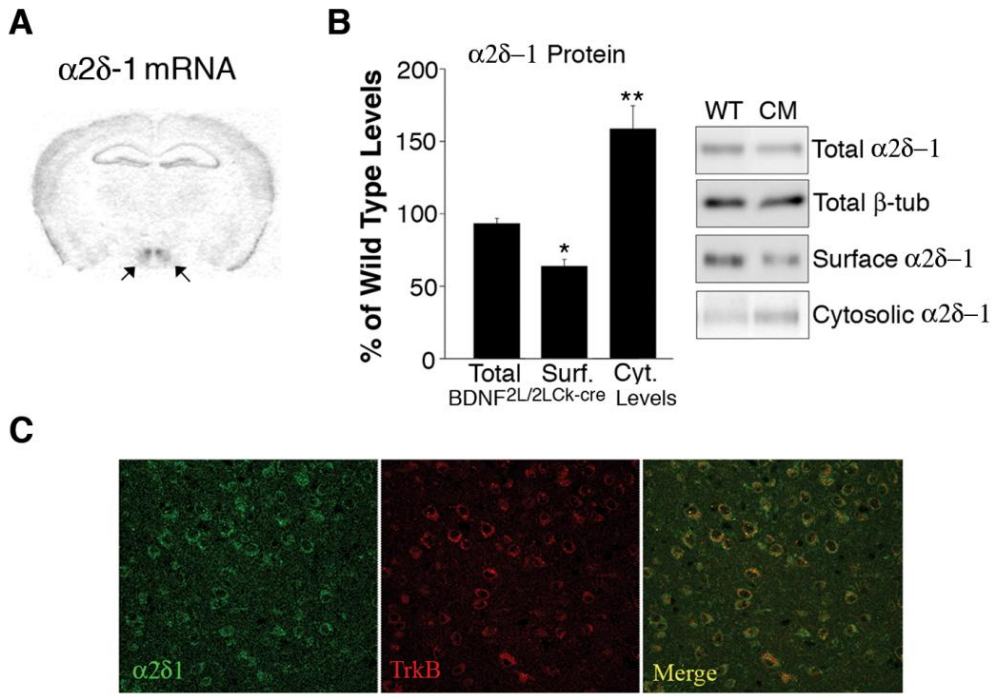

Figure 2.1

Cell surface expression of α2δ-1 is reduced in the VMH of BDNF$^{2L/2LCk\text{-}cre}$ mice. (A) Representative *in situ* hybridization showing high α2δ-1 mRNA expression in the wild type VMH (arrows). **(B)** Western blot analysis of total, surface, and cytosolic α2δ-1 protein content in the VMH of BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant (CM) mice compared to wild type (WT) controls (n=6). *, p = 0.002; **, p = 0.02. **(C)** Confocal images of WT VMH co-immunolabeled with anti- α2δ-1(green) and ant-TrkB (red).

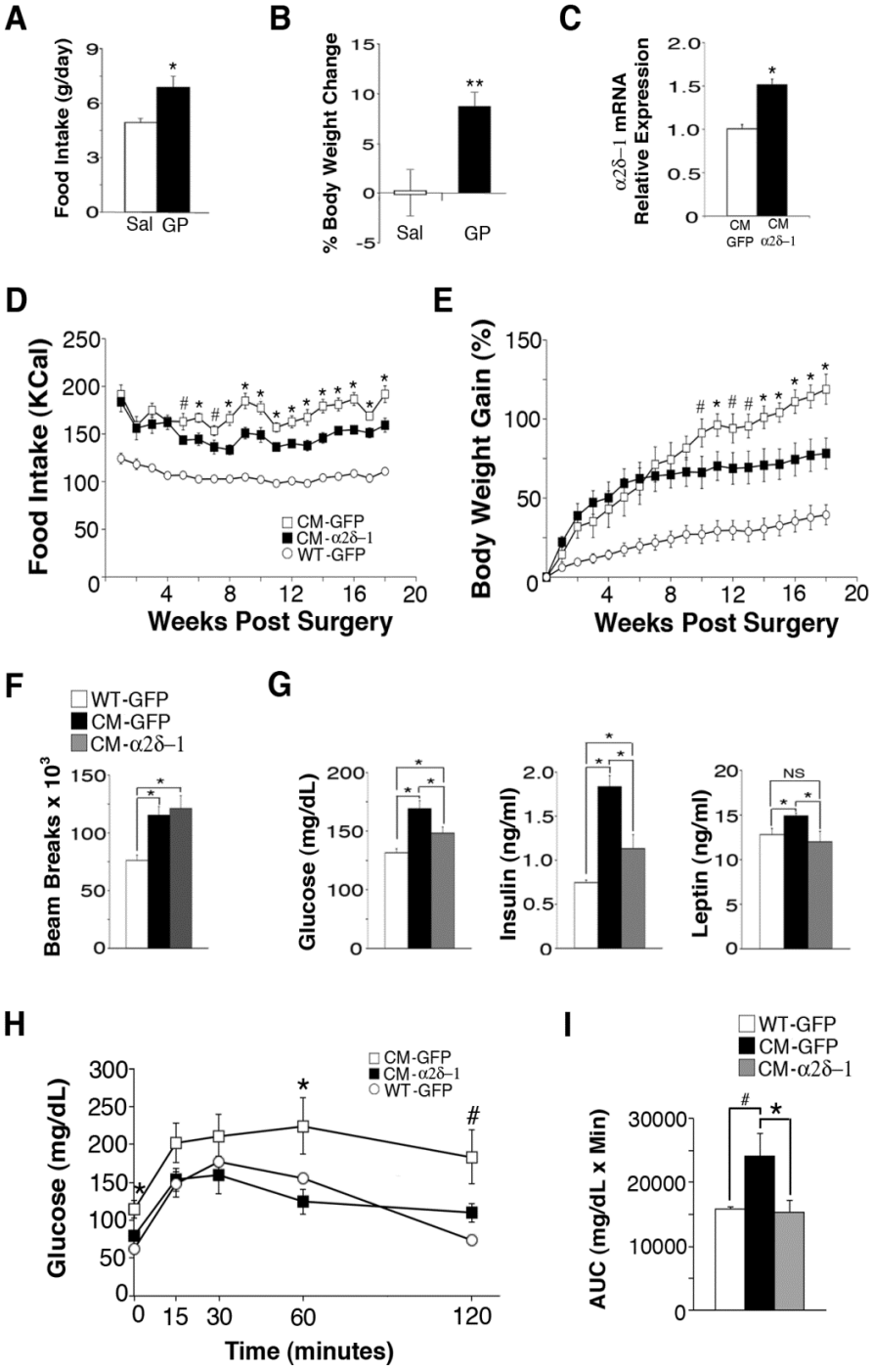

Figure 2.2

α2δ-1 is a novel regulator of appetite acting in the VMH.

Figure 2.2

α2δ-1 is a novel regulator of appetite acting in the VMH. Food intake **(A)** and relative body weight changes **(B)** in WT mice with chronic delivery of saline (Sal) (n=5) or gabapentin (GP) (n=7) to the VMH. *, $p = 0.02$; **, $p = 0.04$. **(C)** Quantitative RT PCR analysis of α2δ-1 mRNA content in the VMH of $BDNF^{2L/2LCk\text{-}cre}$ mice delivered AAV- α2δ-1 (CM- α2δ-1) relative to BDNF mutants delivered AAV-GFP (CM-GFP) to the VMH. *, $p < 0.01$. **(D)** Weekly food intake of CM-GFP, CM-α2δ-1 and WT mice delivered AAV-GFP to the VMH (WT-GFP). There was an effect of treatment on food intake ($F_{(1,10)} = 14.1$ $p < 0.004$, 2-way ANOVA with repeated measures) (n = 6), indicating that the α2δ-1 rescue attenuated the overeating of $BDNF^{2L/2LCk\text{-}cre}$ mutants. *, $p < 0.05$, #, $p < 0.09$, CM-α2δ-1 relative to CM-GFP. There was also a significant effect of treatment on relative body weight gain **(E)** after six weeks ($F_{(1,10)} = 4.78$ $p < 0.05$). *, $p < 0.05$, #, $p < 0.09$, CM-α2δ-1 relative to CM-GFP. **(F)** Measurement of locomotor activity in the home cage over three days expressed as total beam breaks. *, $p < 0.01$. **(G)** Fasted circulating levels of glucose, insulin and leptin in WT-GFP, CM-GFP and CM-α2δ-1 mice. *, $p < 0.04$; ns, not significant. **(H)** Glucose tolerance test showing compromised responses in CM-GFP mice compared to WT-GFP mice ($F_{(1,9)} = 5.10$ $p = 0.05$, 2-way ANOVA with repeated measures) and improved glucose metabolism in CM- α2δ-1 mice compared to the CM-GFP group ($F_{(1,9)} = 5.73$ $p = 0.04$, 2-way ANOVA with repeated measures). *, $p = 0.03$; #, $p = 0.06$. **(I)** Comparison of area under the curve (AUC) for the glucose tolerance test. *, $p = 0.03$; #, $p = 0.07$.

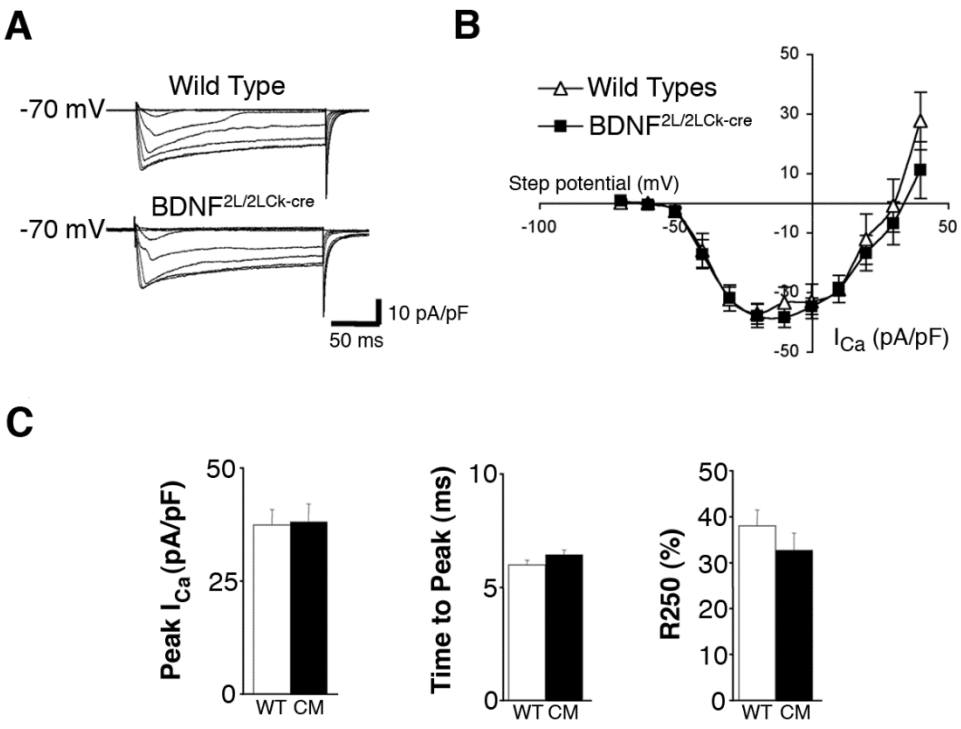

Figure 2.3

Calcium currents in VMH cells of BDNF$^{2L/2LCk\text{-}cre}$ mice are normal. (A) Representative whole cell Ca^{2+} currents recorded from WT and BDNF$^{2L/2LCk\text{-}cre}$ mice. 250-ms test pulses from a holding potential of -70 mV were applied in 10mV increments. **(B)** I-V relationships resulting from step potentials from -70mV to +40mV in 10 mV increments. There was no significant difference in the voltage dependence or amplitude of the Ca^{2+} currents between genotypes (wild type 37.36 ± 3.412 pA/pF; mutant 38.10 ± 3.879 pA/pF; p = 0.888). **(C)** There were no significant difference in the amplitude, or kinetics of Ca^{2+} currents in wild type (WT) and BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant (CM) mice. Histograms for peak current (pA/pF, p = 0.8), time to peak (ms, p = 0.35), and mean inactivation of the maximal current traces at the end of the 250-ms depolarization (R250 %; p = 0.18).

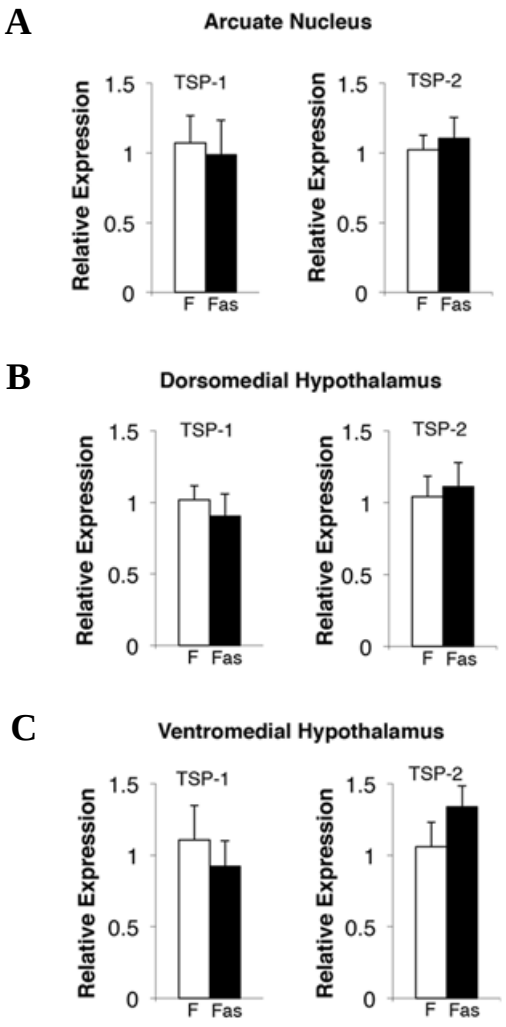

Figure 2.4

TSP-1 and TSP-2 transcripts are not altered by energy status. Quantitative RT-PCR analysis of TSP-1 and TSP-2 mRNA expression levels in the **(A)** arcuate nucleus, **(B)** dorsomedial, and **(C)** ventromedial hypothalamus of fed (f) and fasted (fas) wild type mice revealed no significant differences (n=5).

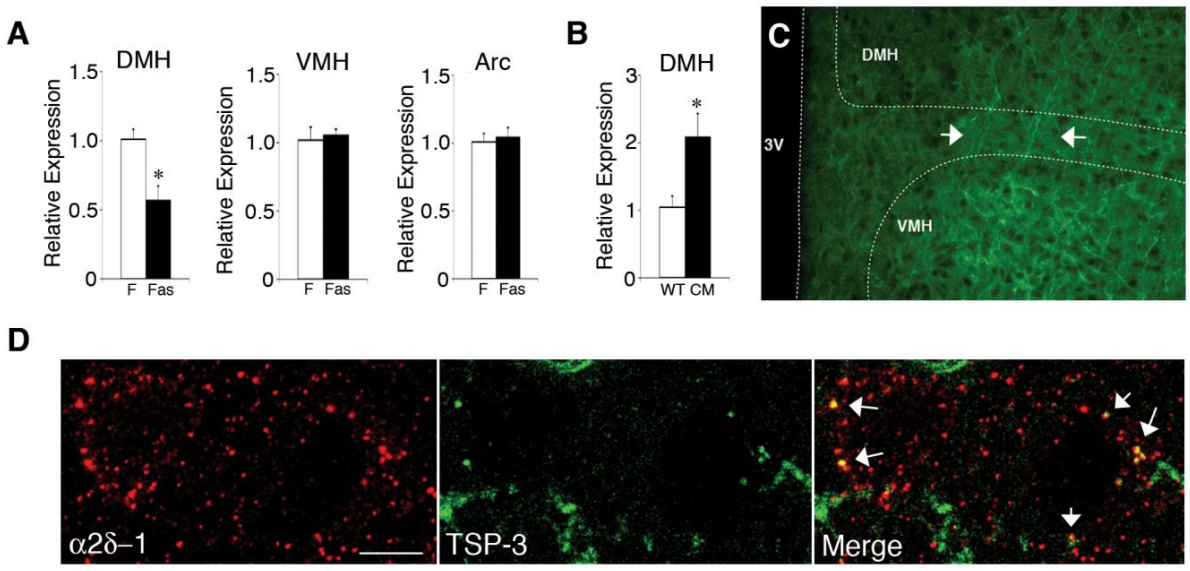

Figure 2.5

Hypothalamic expression of the α2δ-1 ligand, TSP-3 is regulated by energy status signals. **(A)** Quantitative RT-PCR analysis of laser captured cells shows that expression of TSP-3 mRNA is reduced in the DMH but not the VMH or Arc of fasted (Fas) relative to fed (F) wild type mice. *, $p = 0.01$, n = 5. **(B)** Levels of TSP-3 mRNA are significantly elevated in the DMH of BDNF mutant mice. *, $p = 0.03$, n = 5 **(C)** Immunolabeling of wild type hypothalamic sections with anti-TSP-3 shows fibers traveling between the DMH and VMH (arrows). **(D)** Confocal images of non-permeabilized wild type brain sections immunolabeled with anti-α2δ-1 and anti-TSP-3. They show TSP-3 signal in close apposition with α2δ-1 receptors (arrows) in cells in the VMH. Scale bar = 6.5 μM

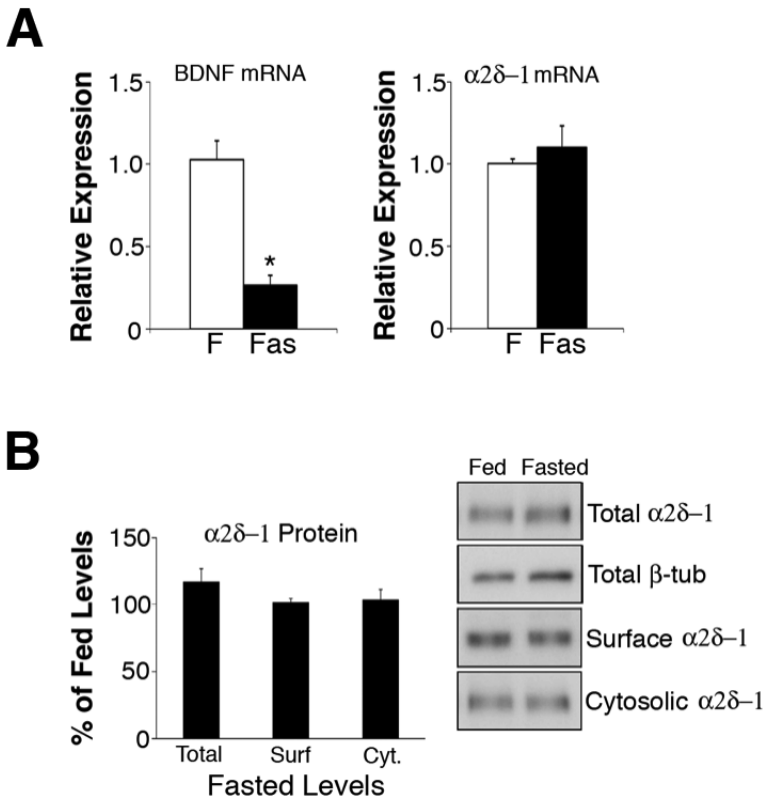

Figure 2.6

Energy status cues do not affect transcript or protein expression of α2δ–1 in the VMH. (A) qRT-PCR analysis showing BDNF and α2δ–1 mRNA expression in the VMH of fed (F) and fasted (Fas) wild type mice. n = 6, *, p = 0.01. (B) Total, cell surface (Surf) and cytosolic (Cyt.) levels of α2δ–1 in the VMH of fasted relative to fed wild type mice (n = 6).

Gene Symbol	Gene Name	Fold Change	FDR (q-value)
Upregulated Genes			
NSG2	neuron specific gene family member 2	**11.834**	0.000
PMCH	pro-melanin-concentrating hormone	**11.025**	0.021
NR4A2	nuclear receptor subfamily 4, group A, member 2	**10.406**	0.015
Downregulated Genes			
BDNF	brain-derived neurotrophic factor	**−15.991**	0.000
TTR	Transthyretin	**−10.028**	0.001
OGN	Osteoglycin	**−9.451**	0.000
CNTN2	contactin 2 (axonal)	**−7.636**	0.000
SLC47A1	solute carrier family 47, member 1	**−6.108**	0.002
SPHK1	sphingosine kinase 1	**−5.683**	0.003
SLC22A2	solute carrier family 22 (organic cation transporter), member 2	**−5.607**	0.003
CRABP2	cellular retinoic acid binding protein 2	**−5.322**	0.005
KCNJ13	potassium inwardly-rectifying channel, subfamily J, member 13	**−5.270**	0.004

C2orf40	chromosome 2 open reading frame 40	**−5.268**	0.005
SLC13A4	solute carrier family 13 (sodium/sulfate symporters), member 4	**−4.906**	0.006
SLC6A13	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	**−4.905**	0.006
C2	complement component 2	**−4.835**	0.007
CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	**−4.722**	0.005
PIGT	phosphatidylinositol glycan anchor biosynthesis, class T	**−4.550**	0.008
THBD	Thrombomodulin	**−4.358**	0.008
FMOD	Fibromodulin	**−4.102**	0.013
FZD7	frizzled homolog 7 (Drosophila)	**−3.993**	0.008
AGRP	agouti related protein homolog (mouse)	**−3.954**	0.031
ALDH1A2	aldehyde dehydrogenase 1 family, member A2	**−3.901**	0.018
PCOLCE	procollagen C-endopeptidase enhancer	**−3.862**	0.020
FOXC2	forkhead box C2 (MFH-1, mesenchyme forkhead 1)	**−3.858**	0.018
GSTK1	glutathione S-transferase kappa 1	**−3.850**	0.008
SLC22A8	solute carrier family 22 (organic anion	**−3.789**	0.017

	transporter), member 8		
Scd2	stearoyl-Coenzyme A desaturase 2	**−3.788**	0.017
ZIC1	Zic family member 1 (odd-paired homolog, Drosophila)	**−3.764**	0.017
PTPRC	protein tyrosine phosphatase, receptor type, C	**−3.702**	0.020
PRG4	proteoglycan 4	**−3.684**	0.024
BMP6	bone morphogenetic protein 6	**−3.566**	0.026
RBM5	RNA binding motif protein 5	**−3.502**	0.017
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	**−3.461**	0.025
GJB2	gap junction protein, beta 2, 26kDa	**−3.442**	0.032
COL3A1	collagen, type III, alpha 1	**−3.427**	0.024
PNPT1	polyribonucleotide nucleotidyltransferase 1	**−3.408**	0.032
TNFSF13	tumor necrosis factor (ligand) superfamily, member 13	**−3.348**	0.024
SLC16A9	solute carrier family 16, member 9 (monocarboxylic acid transporter 9)	**−3.301**	0.025
GFAP	glial fibrillary acidic protein	**−3.287**	0.024
PRKG1	protein kinase, cGMP-dependent, type I	**−3.281**	0.024

MYOC	myocilin, trabecular meshwork inducible glucocorticoid response	**−3.265**	0.058
SLC26A7	solute carrier family 26, member 7	**−3.256**	0.025
SLC13A3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	**−3.118**	0.031
ISLR	immunoglobulin superfamily containing leucine-rich repeat	**−3.104**	0.032
MRC2	mannose receptor, C type 2	**−3.100**	0.033
SPRED3	sprouty-related, EVH1 domain containing 3	**−3.083**	0.023
ST8SIA2	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2	**−3.051**	0.024
CYTL1	cytokine-like 1	**−3.035**	0.025
BICC1	bicaudal C homolog 1 (Drosophila)	**−3.029**	0.032
SLC6A20	solute carrier family 6 (proline IMINO transporter), member 20	**−2.992**	0.040
PCDH15	protocadherin-related 15	**−2.964**	0.032
Plscr2	phospholipid scramblase 2	**−2.933**	0.028
PTGDS	prostaglandin D2 synthase 21kDa (brain)	**−2.925**	0.037
AHNAK	AHNAK nucleoprotein	**−2.924**	0.047
OSMR	oncostatin M receptor	**−2.918**	0.040

COL1A2	collagen, type I, alpha 2	**−2.895**	0.047
MPZL2	myelin protein zero-like 2	**−2.867**	0.048
SLCO4A1	solute carrier organic anion transporter family, member 4A1	**−2.864**	0.032
SPP1	secreted phosphoprotein 1	**−2.852**	0.047
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	**−2.849**	0.031
MGP	matrix Gla protein	**−2.849**	0.049
COL6A3	collagen, type VI, alpha 3	**−2.848**	0.049
Ifitm1	interferon induced transmembrane protein 1	**−2.827**	0.048

Table 2.1 List of differentially expressed genes in the VMH of $BDNF^{2L/2LCk\text{-}cre}$ mice compared to wild type controls revealed by Genechip analysis. A total of 64 differentially expressed genes with a false discovery rate of < 0.05 were identified.

Chapter 3

Brain-derived neurotrophic factor regulates hedonic feeding by acting on the mesolimbic dopamine system

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Abstract

Brain-derived neurotrophic factor (BDNF) and its receptor, TrkB, play prominent roles in food intake regulation through central mechanisms. However, the neural circuits underlying their anorexigenic effects remain largely unknown. We showed previously that selective BDNF depletion in the ventromedial hypothalamus (VMH) of mice resulted in hyperphagic behavior and obesity. Here, we sought to ascertain whether its regulatory effects involved the mesolimbic dopamine system, which mediates motivated and reward seeking behaviors including consumption of palatable food. We found that expression of BDNF and TrkB mRNA in the ventral tegmental area (VTA) of wild-type mice was influenced by consumption of palatable, high-fat food (HFF). Moreover, amperometric recordings in brain slices of mice depleted of central BDNF uncovered marked deficits in evoked release of dopamine in the nucleus accumbens (NAc) shell and dorsal striatum but normal secretion in the NAc core. Mutant mice also exhibited dramatic increases in HFF consumption, which were exacerbated when access to HFF was restricted. However, mutants displayed enhanced responses to D1 receptor agonist administration, which normalized their intake of HFF in a 4 h food intake test. Finally, in contrast to deletion of *Bdnf* in the VMH of mice, which resulted in increased intake of standard chow, BDNF depletion in the VTA elicited excessive intake of HFF but not of standard chow and increased body weights under HFF conditions. Our findings indicate that the effects of BDNF on eating behavior are neural substrate-dependent and that BDNF influences hedonic feeding via positive modulation of the mesolimbic dopamine system.

Introduction

Brain-derived neurotrophic factor (BDNF) and its receptor, TrkB, mediate neuronal survival, differentiation, and plasticity and promote satiety through central mechanisms. Perturbed BDNF signaling triggers hyperphagia and dramatic obesity in mice (Kernie et al., 2000; Lyons et al., 1999; Rios et al., 2001; Xu et al., 2003). In humans, BDNF haploinsufficiency was linked to elevated food intake and obesity (J. Gray, Yeo, Cox, Morton, Adlam, Keogh, Yanovski, El Gharbawy, Han, Tung, Hodges, Raymond, O'rahilly, & Farooqi, 2006b; Han et al., 2008). Even though the cumulative data support a pivotal role of BDNF in feeding control, the neural circuits underlying its regulatory effects remain largely unknown. Food intake is a complex behavior resulting from interactions between homeostatic and reward-related processes. Consistent with a role in homeostatic mechanisms acting in the hypothalamus and hindbrain, energy status influences levels of expression of BDNF and TrkB in these brain regions (Bariohay et al., 2005; Tran, Akana, Malkovska, Dallman, Parada, & Ingraham, 2006a; Unger et al., 2007; Xu et al., 2003). Additionally, selective *Bdnf* deletion in the ventromedial hypothalamus (VMH) of mice elicited hyperphagia and obesity (Unger et al., 2007). BDNF and TrkB are also expressed in the mesolimbic dopamine system (Numan & Seroogy, 1999; Seroogy et al., 1994), which modulates motivated and reward seeking behaviors, including drug and palatable food consumption. BDNF in this pathway has a demonstrated role in drug addiction and social defeat stress (Berton et al., 2006; Graham et al., 2007). The possibility that it influences eating behavior by acting here remains to be investigated.

Mesolimbic fibers originate in the ventral tegmental area (VTA) and terminate in the nucleus accumbens (NAc) and prefrontal cortex, where they release dopamine in response to

drug and palatable food intake (Bassareo & Di Chiara, 1997; Bina & Cincotta, 2000; Ghiglieri et al., 1997; Leddy et al., 2004; M. D. Lee & Clifton, 2002). Appetite-regulating factors, including leptin, ghrelin, and melanin-concentrating hormone regulate mesolimbic dopaminergic activity and some of their effects on feeding involve this system (Abizaid et al., 2006; Fulton et al., 2006; Georgescu et al., 2005; Hommel et al., 2006). BDNF is expressed in dopamine containing cells in the VTA and is anterogradely transported to the NAc, a region of minimal BDNF expression (Conner et al., 1997; Numan & Seroogy, 1999). TrkB, for its part, is localized to VTA dopamine cells and GABAergic medium spiny projection neurons in the NAc (Freeman, Soghomonian, & Pierce, 2003; Numan & Seroogy, 1999; Yan et al., 1997). While BDNF is not essential for survival of VTA dopamine neurons (Baquet, Bickford, & Jones, 2005), it is unclear whether dopamine secretion by these cells requires neurotrophin action.

We investigated whether BDNF in the mesolimbic pathway influenced feeding behavior. We found that mutant mice depleted of central BDNF exhibited marked decreases in evoked release of dopamine in the NAc and dorsal striatum. Furthermore, they displayed excessive intake of palatable food that was normalized by D1 receptor stimulation. Notably, VTA-specific deletion of *Bdnf* resulted in increased feeding and body weight when mutants received a palatable high-fat diet but minimal effects when administered standard chow. These findings indicate that BDNF regulates hedonic feeding by positively modulating the mesolimbic dopamine system.

Materials and Methods

Animals: All of the following procedures were approved by the Institutional Animal Care and Use Committee at Tufts University and were in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals used in these studies and their suffering. $BDNF^{2L/2LCk\text{-}cre}$ mice were generated as previously described and were in a hybrid background with C57BL/6 and 129 strain contributions (Chan, Unger, Byrnes, & Rios, 2006; Rios et al., 2001; Rios et al., 2006). All animals were females between 10 and 12 weeks of age at the start of each experiment. They were fed *ad libitum*, unless otherwise noted, and housed individually in the Tufts University animal care unit on a 12 h light/dark cycle.

Food intake measurements: Food intake of female $BDNF^{2L/2LCk\text{-}cre}$ and wild-type mice (10–12 weeks of age) that had unrestricted access to standard (SC; 5% Kcal Fat, Tekland Global Diet) or palatable high-fat chow (HFF; 45% Kcal Fat, Research Diets) was measured over 3 d of testing. Mice were individually housed and given premeasured amounts of food such that caloric intake could be calculated based on grams of food consumed measured at 2:00 P.M. daily (SC = 3.3 Kcal/g; HFF = 4.7 Kcal/g). Consumption of palatable high-fat chow was also measured when mice had restricted access to this diet. For this, fed female $BDNF^{2L/2LCk\text{-}cre}$ and wild-type mice (10–12 weeks of age) that were individually housed and maintained on a standard chow diet were presented with HFF for 1 h daily (2:00 –3:00 P.M.) for three consecutive days. Daily intake of HFF during the restricted period was measured.

Quantitative reverse transcription-PCR analysis: On day 1 of the experiment, SC or HFF was placed in the cages of naive, individually housed, fed wild-type mice (females, 10–

12 weeks of age) for 60 min to minimize novelty effects. On day 2, following 30 or 60 min of SC or HFF exposure and consumption, mice were killed. Their brains were then rapidly extracted and tissue punches of NAc and VTA obtained from 500-μm thick coronal sections prepared with a vibrating microtome (Leica VT100S). Samples were immediately frozen on dry ice and RNA extracted using Tri Reagent (Molecular Reasearch Center). RNA samples were treated with DNase and tested for genomic DNA contamination in PCRs. Reverse transcription to generate cDNA was conducted with 1 μg of RNA and using 200 units of Superscript II reverse transcriptase (Invitrogen) and 150 ng of random primers (Invitrogen) in a 20 μl reaction. Real time PCR amplification was performed using a MX-3000P Stratagene cycler and SYBR green PCR master mix (Qiagen). For each primer set, the specificity of the product amplification was confirmed by dissociation curve analysis and agarose gel electrophoresis. Furthermore, curves were created using serial dilutions and the efficiencies for each primer set was calculated. The amplification efficiency for all the primers used in this study was >90%. For each target primer set, a validation experiment was performed to demonstrate that the PCR efficiencies were approximately equal to those of the reference gene. A two-step protocol was used: 95°C for 10 min and 45 cycles with 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Actin was used as a normalizer. The following primers were used:

BDNF:	(F) 5'GAAAGTCCCGGTATCCAAAG3',
	(R) 5'CCAGCCAATTCTCTTTTT3';
TrkB:	(F) 5'CCTCCACGGATGTTGCTGAC3',
	(R) 5'GCAACATCACCAGCAGGCA3';
Actin:	(F) 5'GGCTGTATTCCCCTCCATCG3',
	(R) 5'CCAGTTGGTAACAATGCCATGT3'.

All samples were analyzed in triplicates, and nontemplate controls were included to ascertain any level of contamination. Data obtained were analyzed using the comparative Ct method. Every experiment was repeated at least once.

HPLC analysis: Fed female $BDNF^{2L/2LCk\text{-}cre}$ and wild-type mice (10–12 weeks of age) maintained on a standard chow diet were killed and their brains rapidly extracted. NAc and dorsal striatum tissue punches were obtained from coronal sections (500μm) prepared with a vibrating microtome (Leica VT100S). Samples were immediately frozen on dry ice. HPLC for biogenic amines was performed by the Neurochemistry Core Facility at Vanderbilt University.

Western blot analysis: Protein was extracted from NAc and dorsal striatum tissue punches from wild-type and $BDNF^{2L/2LCk\text{-}cre}$ female mice (10–12 weeks of age) and 50μg of each protein extract were separated in an 8% acrylamide gel and transferred to Immuno-blot PVDF membranes (Bio-Rad). Membranes were blocked in a 5% milk TBS-Tween solution and probed individually with antibodies against dopamine transporter and dopamine receptors 1 and 2 (1:1000, Millipore Bioscience Research Reagents) overnight at 4°C. Membranes were then washed, incubated with peroxidase-conjugated secondary antibodies, reacted in ECLplus solution (GE Healthcare) for 5 min, and exposed to Kodak maximum sensitivity film. Densitometry of blots was performed using the Kodak 1D Image analysis software program. Membranes were also hybridized to anti-β-tubulin (Sigma) to normalize the data for loading.

Amperometry: Acute brain slices (300 μm thick) containing NAc and dorsal striatum were prepared from wild-type and $BDNF^{2L/2LCk\text{-}cre}$ mice (females 8–10 weeks of age) using a Leica vibratome. Disk carbon fiber electrodes of 5μm in diameter with a freshly cut surface

were placed ~50 μm into the slice. Wild-type and mutant slices (n = 8) were electrically stimulated (400 μA, 1 ms) with a bipolar stimulating electrode (Plastics One) placed ~100 μm from the recording electrode using an Iso-Flex stimulus isolator (A.M.P.I.) triggered by a pulse generator (A.M.P.I.). Background-subtracted cyclic voltammograms serve to calibrate the electrodes and to optimize the identification of the released substance as dopamine.

Pharmacology: Food-nondeprived $BDNF^{2L/2LCk\text{-}cre}$ and wild-type mice were individually housed and maintained on standard chow. At the beginning of the experiment, 1:00 P.M., an intraperitoneal injection of R(±)SKF-38393 (20 mg/kg; Sigma-Aldrich) or saline (0.9% NaCl) was administered. Upon injection, mice were given premeasured amounts of HFF such that grams of food consumed and caloric intake (4.7 kcal/g) could be calculated at 1/2, 1, 2, 3, and 4 h after injection. Mice were habituated to experimental conditions by gentle handling and saline injections for 3 d preceding the experiment. One day before drug administration, mice were given 4 h of access to HFF to reduce novelty effects.

Deletion of* Bdnf *in the VTA: AAV2/1 vectors were produced, purified, and titered as previously described (Broekman et al., 2006). Cre recombinase cDNA carrying an N-terminal nuclear localization signal was cloned into the AAV2-CBA-W vector plasmid. Adeno-associated virus (0.5 μl; 1 x 10^{13} infectious units per ml) encoding Cre recombinase (AAV2/1-Cre) or green fluorescence protein (AAV2/1-GFP) was delivered to the VTA of anesthetized female floxed *Bdnf* mice (10–12 weeks of age) using the coordinates: anteroposterior - 3.2 mm, mediolateral, ± 1.0 mm, and dorsoventral, - 4.6 mm at 10 degrees from midline. The virus was administered bilaterally using a 10 μl Hamilton syringe with a 33-gauge needle attached to a digital stereotaxic apparatus (Benchmark) and an infusion

microsyringe nanopump (KD Scientific) at a rate of 0.15 μl/min. To allow diffusion of the virus and minimize backflow after needle retraction, the needle was held in place for 10 min after injection. Mice were given a 1 week recovery from surgery before any experiments were conducted. Viral volume and coordinates for selectively targeting the VTA were optimized using the mouse atlas (Paxinos and Franklin, 2001) and by pilot experiments injecting AAV2/1-Cre into ROSA reporter mice (Soriano, 1999). Accurate targeting was confirmed in AAV2/1-GFP mice by analysis of GFP signal and in AAV2/1-Cre-injected mice by measurement of BDNF mRNA expression (*in situ* hybridization analysis).

In situ *hybridization analysis:* Twelve-micrometer-thick sections containing VTA from wild-type, $BDNF^{2L/2LCk\text{-}Cre}$ mutant, and AAV2/1- GFP- and AAV2/1-Cre-injected mice were hybridized for 16 h at 60°C with a 35S-labeled, antisense riboprobe representing bases 507–833 of the BDNF cDNA. Specificity of this riboprobe was confirmed by lack of hybridization to tissue from $BDNF^{2L/2LCk\text{-}Cre}$ mutant mice (Rios et al., 2001). Following the hybridization step, sections were stringently washed and placed on x-ray film for 12 d. Densitometry of BDNF mRNA signal was conducted using the Kodak 1D Image Analysis software. Coronal level was confirmed by staining adjacent sections with cresyl violet.

Locomotor activity measurements: Mice were housed individually in standard 15 × 24 cm plastic cages on a 12 h reverse light/dark cycle. Motor activity was monitored with the Smart Frame Activity System (Hamilton/Kinder), consisting of 12 PC-interfaced horizontal photobeam frames (8 cm length × 4 cm width spaced 1.5 cm apart) that surrounded the animal's home cage. At the start of the experiment, 10:00 A.M., an intraperitoneal injection either saline (0.9% NaCl), SKF- 81297 (5 mg/kg; Sigma-Aldrich), or Quinpirole (2.5 mg/kg; Sigma- Aldrich) was administered in a volume of 10 μl per gram

body weight. Locomotor readouts (beam breaks) were subsequently recorded for 3 h using MotorMonitor software (Hamilton/Kinder). During the entire testing period, animals remained undisturbed. Mice were habituated to experimental conditions by gentle handling and saline injections for 3 d preceding the experiment.

Statistical analysis: Food intake measurements for experiments involving $BDNF^{2L/2LCk\text{-}Cre}$ mice were analyzed using two-way ANOVA. Amperometric recordings were analyzed using a one-way ANOVA. Quantification of *in situ* hybridizations, quantitative reverse transcription (RT)-PCR, Western blot analysis and food intake and body weights of AAV2/1-GFP- and AAV2/1-Cre-injected mice were analyzed by unpaired *t* test. Data were considered statistically significant when $p < 0.05$ and all values represent mean ± SEM.

Results

BDNF conditional mutant mice exhibit increased intake of palatable food

The mesolimbic dopamine system is linked to appetitive motivation and consumption of highly palatable food (Berridge, 2009). As a first step to evaluate how BDNF activity in this pathway might influence eating behavior, we measured consumption of palatable HFF in mice with central depletion of BDNF ($BDNF^{2L/2LCk\text{-}cre}$). Mutants were generated by crossing mice carrying floxed *Bdnf* alleles with transgenic mice expressing cre recombinase under the control of the α-calcium/calmodulin protein kinase II promoter and were described previously (Chan et al., 2006; Rios et al., 2001; Rios et al., 2006). In these animals, BDNF expression was terminated across the brain, the cerebellum exempted. Depletion of BDNF

began during the first postnatal week and became maximal at 3 weeks after birth. To confirm that BDNF mRNA was depleted in the VTA of $BDNF^{2L/2LCk\text{-}cre}$ mutants, we conducted *in situ* hybridization analysis. As indicated in supplemental Figure 3.1, BDNF mRNA was extensively depleted from the VTA of mutants.

We reported previously that when fed SC, $BDNF^{2L/2LCk\text{-}cre}$ mutants exhibited hyperphagic behavior and became dramatically obese (Rios et al., 2001). Here, we examined whether they also exhibited higher levels of consumption of palatable HFF. We also measured SC consumption for comparison. There was an effect of genotype on food intake ($F_{(1,18)} = 75.6$, $p = 0.000001$). Whereas $BDNF^{2L/2LCk\ cre\ mice}$ fed SC *ad libitum* consumed 83% more kilocalories than WT mice fed SC ($p = 0.0001$), their caloric intake was elevated by 111% ($p = 0.0001$) when they had unrestricted access to HFF compared with wild-types fed a similar diet (Fig. 3.1*A*).

Restricted access to palatable food elicits binge eating behavior in wild-type rodents (Berner, Avena, & Hoebel, 2008; Corwin & Wojnicki, 2006; Davis et al., 2007). We asked whether this effect was exaggerated in $BDNF^{2L/2LCK\text{-}cre}$ mutant mice. Fed wild-type and BDNF mutant mice maintained on a SC diet had access to palatable HFF for 1 h/d for 3 consecutive days. Food intake during the restricted access period was measured. HFF caloric intake of BDNF mutant mice during the restricted period was increased by 214% ($p < 0.0001$), 209% ($p < 0.0001$), and 200% ($p < 0.0001$) on days 1, 2, and 3, respectively, compared with wild-type mice (Fig. 3.1*B*). The collective behavioral data indicate that central depletion of BDNF induces dramatic hyperphagic behavior when animals have free access to palatable HFF. Furthermore, limited exposure to HFF exacerbates the binge-eating behavior triggered by deficient BDNF signal.

Levels of TrkB and BDNF mRNA in the VTA are influenced by intake of palatable food

To investigate whether BDNF signaling in the mesolimbic system might influence feeding behavior, we examined the effect of palatable food ingestion on its expression and that of its receptor, TrkB, in the VTA and NAc of sated wild-type mice. Following 30 or 60 min of SC or HFF exposure and consumption, mice were killed and VTA and NAc tissue punches obtained for quantitative RT-PCR analysis. We found that TrkB mRNA expression in the NAc of SC and HFF-fed mice was similar at both time points (data not shown). In the VTA, there was a 46% increase ($p = 0.04$) in TrkB receptor transcript content following 30 min of HFF consumption and this increase returned to baseline levels after 1 h of palatable food intake (Fig. 3.2*A*). In contrast, BDNF expression in the VTA remained stable at 30 min but was decreased by 38% ($p = 0.004$) following 60 min of HFF consumption (Fig. 3.2*B*). These data indicate that expression of TrkB and BDNF mRNA in the VTA is influenced by consumption of palatable food and that BDNF signaling in this region is finely regulated during food intake-related processes.

BDNF conditional mutants exhibit deficits in evoked release of dopamine in the nucleus accumbens shell and dorsal striatum

Because $BDNF^{2L/2LCk\text{-}cre}$ mutant mice exhibited increased intake of palatable HFF, which influenced expression of BDNF and TrkB mRNA in the VTA, we sought to ascertain whether alterations in the mesolimbic dopamine system might underlie their excessive eating. First, we evaluated dopamine synthesis and turnover in the nucleus accumbens of wild-type

and BDNF mutant mice. The dorsal striatum was also examined for comparison. We found no significant changes in content of dopamine or its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the NAc of BDNF mutant mice (Fig. 3.3*A*). However, there was an 18% increase ($p = 0.02$) in the DOPAC/ dopamine (DA) ratio in the mutants compared with wild-types, indicating a mild elevation in dopamine turnover. In the dorsal striatum, $BDNF^{2L/2LCk\text{-}cre}$ mutant mice exhibited 22% ($p = 0.002$) and 40% ($p = 0.007$) increases in dopamine and DOPAC content, respectively, but normal DOPAC/DA ratios compared with wild-type mice (Fig. 3.3*B*).

Next, we performed amperometric recordings to determine whether BDNF influenced dopamine release through pre-synaptic mechanisms. We measured electrically evoked secretion of dopamine in acute brain slices obtained from wild-type and $BDNF^{2L/2LCk\text{-}cre}$ mutant mice. Amperometric recordings were obtained from dopamine terminals in the NAc shell and core that originated in the VTA. Deficient evoked release of dopamine was evident in the NAc shell but not in the NAc core of BDNF mutants (Fig. 3.4*A*–*D*). The mean evoked dopamine signal amplitude in the NAc shell of BDNF mutants was decreased by 39% (18.4 ± 1.5 pA; $n = 68$ stimulations in 14 slices) compared with wild-types (30.1 ± 2.7 pA; $n = 70$ stimulations in 15 slices) ($F_{(1,136)} = 14.15$, $p < 0.01$) (Fig. 3.4*C*). This difference persisted following addition of nomifensine, a dopamine transporter (DAT) inhibitor, indicating that the deficit was due to reduced secretion, not to increased reuptake of dopamine. When the number of dopamine molecules released was calculated based on spike amplitude (pA) and width (s), $BDNF^{2L/2LCk\text{-}cre}$ mutant mice exhibited a 43% decrease ($F_{(1,136)} = 17.33$, $p < 0.01$) in dopamine release in the NAc shell compared with controls (Fig. 3.4*B*). This deficit persisted in the presence of nomifensine ($F_{(1,117)} = 14.39$, $p < 0.01$) (Fig. 3.4*B*).

Amperometric measurements in the dorsal striatum revealed additional deficits in BDNF mutant mice (Fig. 3.5*A*–*D*). BDNF mutant slices showed a 64% decrease in mean evoked dopamine molecules compared with wild-types (BDNF mutants: $38.5 \times 10^6 \pm 4.0 \times 10^6$, $n = 49$ stimulations in 10 slices; wild-types: $106.3 \times 10^6 \pm 15.6 \times 10^6$, $n = 44$ stimulations in 9 slices; $F_{(1,135)} = 30.60$, $P < 0.01$) (Fig. 3.5*B*). As in the NAc shell, this difference persisted in the presence of nomifensine (Fig. 3.5*B*). Moreover, whereas mutants exhibited a mean signal amplitude of 15.9 ± 2.2 pA ($n = 49$ stimulations in 10 slices), wild-types showed an amplitude of 36.0 ± 5.5 pA ($n = 44$ stimulations in 9 slices) ($F_{(1,135)} = 26.11$, $p = 0.01$) (Fig. 3.5*C*). In artificial CSF (ACSF) with nomifensine, BDNF mutant slices also exhibited a significant decrease in mean signal amplitude, 20.7 ± 3.7 pA ($n = 30$ stimulations in 6 slices) versus 47.3 ± 4.9 ($n = 20$ stimulations in 4 slices) in wild-type slices ($F_{(1,68)} = 40.92$, $p < 0.01$). These data demonstrate that lack of central BDNF triggers an increase in dopamine synthesis in the dorsal striatum and marked decreases in dopamine release in the NAc shell and dorsal striatum.

Expression of dopamine receptors and transporter in the striatum of BDNF conditional mutant mice

To further evaluate dopamine transmission in the absence of central BDNF, we sought to ascertain whether the observed decreases in evoked release of dopamine in BDNF mutants were accompanied by changes in levels of expression of D1 (D1R) and D2 (D2R) receptors or dopamine transporter. We found that expression of D1R, D2R and DAT was normal in the NAc of $BDNF^{2L/2LCk\text{-}cre}$ mutant mice (data not shown). In the dorsal striatum, whereas expression of D1R and DAT was normal in the mutants, expression of D2R was

decreased by 34% (p = 0.03) (Fig. 3.6*A*–*C*). These findings indicate that dopamine receptor and transporter content is normal in the NAc of BDNF mutants despite them having decreased release of dopamine in this region. However, diminished BDNF signal led to decreased D2R expression in the dorsal striatum, reminiscent of the D2R downregulation observed in dietary obese animals and humans (Geiger et al., 2009; Pothos et al., 1998; G. J. Wang et al., 2001).

Treatment with selective D1 receptor agonists normalized consumption of palatable high-fat food in BDNF$^{2L/2LCk\text{-}cre}$ mutant mice

In light of the deficient dopamine release but normal D1R expression observed in the NAc of BDNF mutant mice, we investigated whether D1 receptor stimulation normalized their eating behavior. To assess this, fed wild-type and BDNF$^{2L/2LCk\text{-}cre}$ mutant mice maintained on a SC diet received a peripheral injection of the selective D1R agonist, SKF-38393 (20 mg/kg) and their intake of palatable HFF was monitored for the following 4 h. There was a significant effect of genotype ($F_{(1,16)}$ = 8.72, p < 0.01) and a significant interaction of genotype and drug treatment ($F_{(1,16)}$ = 5.72, p < 0.03) on caloric intake. Remarkably, SKF-38393 treatment had a more profound and persistent effect in BDNF mutants compared with wild-type mice, completely normalizing their cumulative HFF intake (Fig. 7*A*,*B*). During the first hour following drug administration, wild-types ate 80% less than vehicle-treated wild-type controls (p = 0.01) (Fig. 3.7*A*). During that same period of time, BDNF mutants treated with SKF-38393 ate 95% less HFF than vehicle-treated BDNF$^{2L/2LCk\text{-}cre}$ mice (p= 0.01) (Fig. 3.7*A*). There were no significant differences in cumulative food intake after 2 h between vehicle and drug-treated wild-types (Fig. 3.7*A*). In

far contrast, the anorexigenic effect of SKF-38393 persisted in BDNF mutants 2 h after drug treatment as indicated by a 94% reduction in cumulative caloric intake ($p = 0.002$) compared with their vehicle-treated counterparts (Fig. 3.7*A*). When total food consumption during the 4 h following D1R agonist administration was calculated, $BDNF^{2L/2LCk\text{-}cre}$ mice showed a 48% reduction in HFF caloric intake compared with vehicle-treated mutants ($p = 0.04$) (Fig. 3.7*A*,*B*). In contrast, SKF-38393 treatment had no significant effect on the 4 h cumulative HFF intake of wild-types compared with vehicle-treated wild-type controls. (Fig. 3.7*A*,*B*). It is important to note that the 4 h cumulative caloric intake of SKF-38393-treated BDNF mutant mice was indistinguishable from that of SKF-38393 or vehicle treated wild-type mice (Fig. 3.7*A*,*B*). These results show that stimulation of D1 receptors normalizes consumption of palatable HFF in $BDNF^{2L/2LCk\text{-}cre}$ mice, which are hyper responsive to the effects of D1R agonists compared with wild-types. These findings are consistent with deficient dopamine transmission contributing to the abnormal eating behavior triggered by depleted BDNF stores.

Site-specific deletion of *Bdnf* in the VTA of adult mice

To pinpoint the role of BDNF in the mesolimbic reward pathway, we examined the effect of selectively deleting *Bdnf* in the VTA, a principal source of BDNF for the nucleus accumbens (Guillin et al., 2001). VTA-specific mutant and control mice were generated by delivery of adeno-associated viral vectors encoding for cre recombinase (AAV2/1-Cre) or green fluorescent protein (AAV2/ 1-GFP), respectively, to the VTA of floxed *Bdnf* mice. Figure 3.8*A* illustrates a representative study entailing stereotaxic delivery of AAV2/1-Cre to the VTA of Rosa-β-gal reporter mice (Soriano, 1999). Blue cells represent sites where cre

recombinase was expressed to mediate recombination of floxed sequences. Blue cells were evident in the VTA of Rosa-β-gal reporter mice injected with AAV2/1-Cre, indicating that floxed alleles were selectively and efficiently recombined in the VTA. Viral spread was restricted to the VTA with minimal infection of the adjacent substantia nigra, consistent with previous reports of contained infection within targeted brain areas due to large viral particle size and boundaries created by fiber bundles (Berton et al., 2006; Graham et al., 2007; Hommel, Sears, Georgescu, Simmons, & DiLeone, 2003; Hommel et al., 2006). Densitometry of BDNF mRNA signal in the VTA showed that BDNF transcript levels were reduced by an average 68% in AAV2/1-Cre mice compared with AAV2/1-GFP controls (p = 0.004) (Fig. 3.8*B*). Figure 3.8, *C* and *D*, show representative coronal brain sections from floxed *Bdnf* mice injected with AAV2/1-GFP or AAV2/1-Cre, demonstrating selective depletion of BDNF mRNA in the VTA of the latter. Furthermore, expression of BDNF mRNA was intact in the hypothalamus of AAV2/1-Cre injected mice (supplemental Fig. 3.2), ensuring that food intake behavior was not affected by hypothalamic depletion of this neurotrophin. Finally, examination of cresyl violet-stained sections containing VTA and obtained from AAV2/1-GFP and AAV2/1-Cre-injected mice failed to reveal any toxicity effects of the viral injection (data not shown).

AAV2/1-GFP and AAV2/1-Cre-injected mice identified through *post hoc* examination as having correct targeting of the VTA and AAV2/1-Cre-treated animals with BDNF mRNA depletion in the VTA ranging from 60 to 78%were included in our food intake and body weight analysis. Both sets of mice were fed SC *ad libitum* for 10 weeks following viral delivery and then switched to a palatable high-fat diet during the subsequent 10 weeks. AAV2/1- Cre-injected mice fed SC did not exhibit significant changes in food intake

compared with AAV2/1-GFP controls (Fig. 3.8*E*). In contrast, when they were administered palatable HFF after week 10 of the study, their caloric intake was significantly increased compared with AAV2/1 GFP controls (Fig. 3.8*E*). Indeed, during the first week of HFF consumption, AAV2/1-Cre mice ate 23% more than AAV2/1-GFPtreated mice ($p = 0.03$). Furthermore, whereas caloric intake of AAV2/1-GFP treated mice trended toward a significant 16% increase ($p = 0.06$) when they transitioned from a SC to a high-fat diet, VTA BDNF mutants increased their caloric intake by 54% ($p = 0.01$) (Fig. 3.8*E*). Food intake of AAV2/1-Cre-treated mice was also significantly increased at week 4 ($p = 0.05$) of HFF consumption (14 weeks after viral delivery) and trended toward significant increases at weeks 2 (12 weeks after viral delivery) and 10 (20 weeks after viral delivery) of HFF consumption (Fig. 3.8*E*).

Consistent with their food intake behavior under SC conditions, AAV2/1-Cre-injected mice had no significant increases in body weight when fed this diet, except at week 6 after surgery, when they exhibited a 12% increase compared with AAV2/1-GFP controls ($p = 0.04$) (Fig. 3.8*F*). In contrast, AAV2/1-Cre-injected mice became significantly heavier (15% increase compared with wild-types) ($p = 0.02$) after 1 week of HFF consumption and elevated body weights persisted until the end of the study (Fig. 8*F*). After 10 weeks of HFF consumption, AAV2/1-Cre mice were 30% heavier than AAV2/1-GFP controls ($p = 0.003$) (Fig. 3.8*F*).

We also measured locomotor activity of VTA-specific BDNF mutants for 3h following a single injection of saline vehicle, the D1R agonist SKF-81297 (5 mg/kg) or the D2R agonist quinpirole (2.5 mg/kg). Levels of activity of saline-injected AAV2/1- GFP and AAV2/1-Cre mice were similar (supplemental Fig. 3.3). Moreover, D1R and D2R

stimulation elicited increases and decreases, respectively, in locomotor activity in both sets of mice. However, whereas the magnitude of the response to quinpirole was comparable in AAV2/1-GFP and AAV2/1-Cre mice, the response to SKF-81297 appeared to be more pronounced in AAV2/1-GFP controls. D1R stimulation increased locomotor activity by 75% ($p = 0.01$) in AAV2/1-GFP mice and by 42% ($p = 0.01$) in AAV2/1-Cre mutants relative to their corresponding saline controls (supplemental Fig. 3.3). However, an interaction between genotype and drug treatment did not reach statistical significance. The cumulative data from studies involving selective depletion of BDNF in the VTA demonstrate that this neurotrophin is required in this brain region for the regulation of food intake. Moreover, they suggest that BDNF action in the mesolimbic system has a larger impact on hedonic relative to homeostatic feeding.

Discussion

The mesolimbic dopamine system is associated with hedonic mechanisms that drive appetitive behavior and intake of highly palatable, energy-dense food. However, the mechanisms governing this pathway during food consumption-related processes remain poorly understood. The multidisciplinary studies described here indicate an essential role of BDNF in the control of hedonic feeding via positive regulation of mesolimbic dopamine transmission. We showed that selective targeting of *Bdnf* in the VTA of mice caused increased intake of palatable HFF but not of SC. This is in contrast to our previous findings demonstrating that mice depleted of BDNF in the VMH exhibited hyperphagic behavior when fed SC *ad libitum* (Unger et al., 2007). The results also differ from rats with selective RNAi-mediated knockdown of leptin receptors in the VTA, which exhibited increased intake

of SC and higher sensitivity to palatable food (Hommel et al., 2006). Our observations indicate that the effects of BDNF on eating behavior are context dependent and influenced by the neural substrates. Whereas hypothalamic BDNF appears to regulate homeostatic food intake, in the VTA, this neurotrophin appears to largely affect hedonic processes driving palatable food consumption.

Because BDNF was depleted in the VTA of adult mice that had normal BDNF expression throughout development, the observed behavioral alterations cannot be attributed to developmental defects. Instead, they indicate a regulatory role of BDNF in the mature brain. Consistent with this assertion, we found that expression of TrkB and BDNF mRNA in the VTA of sated wild-type mice was influenced by intake of palatable HFF. The site of these expression changes suggests that BDNF might act presynaptically through autocrine mechanisms to modulate dopamine producing cells in the VTA during food reward related processes. Evidentiary is our finding that $BDNF^{2L/2LCk\text{-}cre}$ mice exhibited diminished evoked release of dopamine in the NAc shell, a target site of VTA dopamine neurons. Because HPLC analysis demonstrated normal dopamine content in the mutant NAc, this defect cannot be attributed to deficient dopamine synthesis. Moreover, reduced extracellular dopamine levels persisted in the presence of a DAT inhibitor, indicating that defective presynaptic release rather than enhanced dopamine clearance mediated the observed deficit.

Diminished dopamine secretion was only evident in the NAc shell and not in the NAc core of $BDNF^{2L/2LCk\text{-}cre}$ mutant mice. A divergence in function in these ventral striatal compartments was reported previously. For example, intake of palatable food was reported to induce dopamine release preferentially in the NAc shell compared with the core (Tanda & Di Chiara, 1998). Furthermore, *in vivo* microdialysis studies in rats revealed that whereas

unpredicted consumption of palatable food led to release of dopamine in the shell, food anticipation was related to secretion in the core (Bassareo & Di Chiara, 1999). Directly relevant to the diminished dopamine signal in the NAc shell of $BDNF^{2L/2LCk\text{-}cre}$ mutants is the observation that inhibition of the NAc shell by local infusion of muscimol, a GABAA receptor agonist, resulted in dramatic hyperphagic behavior in animals fed *ad libitum* (Baldo et al., 2005).

Administration of a D1R selective agonist normalized the excessive intake of HFF exhibited by BDNF mutant mice in a 4 h HFF consumption test. Indeed, similar doses of D1R agonist had a far more profound and extended effect in the mutants compared with wild-types. This finding provides further evidence of a link between deficits in dopamine transmission elicited by perturbed BDNF signaling and increased intake of palatable food. It also suggests that homeostatic adaptations take place in BDNF mutants that confer hypersensitivity to D1R stimulation. This notion is supported by studies involving dopamine-deficient mice, which exhibited hypersensitivity to D1R agonists that was ameliorated by 4 d of DOPA treatment (Kim, Szczypka, & Palmiter, 2000). The enhanced responses of $BDNF^{2L/2LCk\text{-}Cre}$ to SKF-38393 were not due to changes in D1R expression in the NAc or dorsal striatum of BDNF mutant mice. It is possible that depleted BDNF might lead to compensatory changes including increased affinity for D1R ligand or enhanced G-protein coupling to D1 receptors, which was demonstrated previously to confer hypersensitivity to dopamine (Gainetdinov et al., 2003).

Deficits in dopamine secretion were not limited to the NAc of BDNF mutant mice. Marked decreases were also observed in the dorsal striatum. Moreover, increased content of dopamine in this region was evident in $BDNF^{2L/2LCk\text{-}cre}$ mice, perhaps indicative of a

compensatory homeostatic response to the reduced extracellular levels of dopamine in the mutants. Dopamine transmission in the dorsal striatum, which is densely innervated by dopaminergic fibers from the substantia nigra, has been linked to food reward processes. Release of dopamine in the dorsal striatum of healthy human subjects was induced by ingestion of palatable food and the amount of dopamine released correlated with the degree of experienced pleasure reported (D. M. Small et al., 2003). Therefore, we cannot rule out the possibility that deficient dopamine signaling in the dorsal striatum of $BDNF^{2L/2LCk\text{-}cre}$ mutants contributes to their excessive intake of palatable food. Nevertheless, it is clear from the studies described here that BDNF produced in the VTA plays a pivotal part in the control of feeding behavior.

BDNF was reported previously to facilitate synaptic sensitization of VTA dopamine neurons following cocaine withdrawal, which might represent a mechanism mediating drug craving and relapse (L. Pu et al., 2006). Moreover, Graham et al. (2007) demonstrated that selective depletion of BDNF in the mesolimbic system resulted in reduced cocaine self-administration, indicating that BDNF promotes the development and persistence of addictive behavior. In contrast, we found that deleting *Bdnf* in the VTA of mice resulted in increased intake of palatable high-fat food, a natural reward. An important distinction between these studies is that Graham et al. targeted *Bdnf* in the NAc of mice and attributed the observed alterations to disrupted BDNF signaling at postsynaptic sites in the NAc. This model was further supported by studies showing that TrkB knock down in the NAc but not in the VTA reduced cocaine reward (Graham et al., 2009). For our studies, we deleted *Bdnf* in the VTA and we postulated that BDNF acts pre-synaptically to positively modulate dopaminergic activity during food reward-related processes. Therefore, the findings from both of these

studies suggest a disassociation of disease mechanisms mediating drug addiction and eating disorders.

Altered dopaminergic transmission has been linked to the emergence of eating disorders and obesity. However, conflicting disease mechanisms have been proposed. One model proposes that a heightened response of the dopaminergic reward circuitry to palatable food underlies excessive eating (Dawe, Gullo, & Loxton, 2004). An alternative model postulates that hypoactivity of this neural system leads to reward deficiency syndrome and behaviorally, to compensatory overeating to boost a deficient dopaminergic system (Blum et al., 2000; Pothos et al., 1998; G. J. Wang, Volkow, & Fowler, 2002). Based on our findings, we propose that perturbed BDNF signaling impedes activity of the mesolimbic dopamine pathway, leading to reward deficiency and compensatory overeating of palatable food. Consistent with this model, it was reported that over expression of Δ-FosB resulted in reduced protein levels of BDNF and concomitant molecular changes in the NAc consistent with deficient dopamine signaling, including reduced levels of DARPP-32 and pCREB (Teegarden, Nestler, & Bale, 2008). Notably, consumption of palatable HFF for 6 weeks completely reversed these abnormalities in Δ-FosB mice.

Rodent studies have shed some light onto putative pathological mechanisms involving dopamine systems and underlying eating disorders. Hyperphagic dietary obese and obesity-prone rats and leptin-deficient *ob/ob* mice exhibited reduced evoked dopamine release in the NAc (Fulton et al., 2006; Geiger et al., 2009; Pothos et al., 1998). Moreover, *ob/ob* mice displayed reductions in food intake and body weights when treated with D1R and D2R agonists (Bina & Cincotta, 2000). Some intriguing evidence comes from studies involving dopamine-deficient mice. They exhibit decreased food intake and without

intervention fail to eat enough to sustain life beyond 48 h (Szczypka, Rainey et al., 1999). However, whereas dopamine production selectively restored in the dorsal striatum of mutants rescued feeding of normal chow, restoration in either the NAc or dorsal striatum led to increased intake of palatable food (Szczypka et al., 2001). Furthermore, when *ob/ob* mice were crossed with dopamine-deficient mice, their hyperphagic behavior was diminished (Szczypka, Rainey, & Palmiter, 2000). These findings in dopamine-deficient mice demonstrate the important role of dopamine in more than one aspect of feeding motivation and a need to compensate for a complete dopamine deficit that might be absent in animals where central dopamine is present but hypofunctioning. The complexity of mechanisms underlying appetite control and the need for additional investigations to resolve these differences are evident. Nonetheless, the studies described here strongly support a pivotal role of BDNF in the positive regulation of mesolimbic dopamine transmission during food reward processes.

In summary, we showed that the mesolimbic dopamine system is a newly identified target of action of BDNF for the control of feeding behavior. Moreover, that BDNF in the VTA has higher relevance in the regulation of hedonic processes impacting consumption of palatable food. Our findings implicate the dopaminergic reward circuit in the disease mechanisms triggered by perturbed BDNF signaling, leading to excessive food intake and obesity.

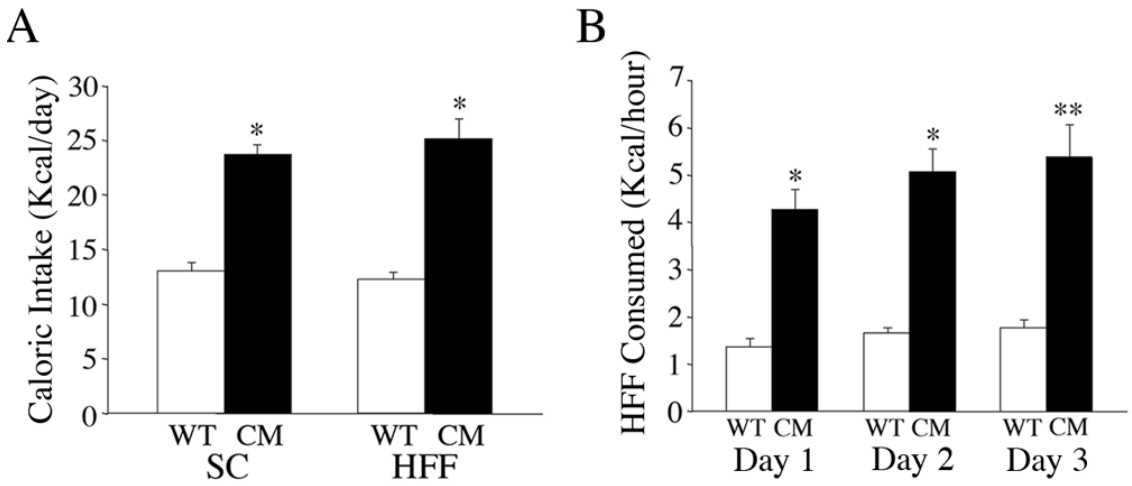


Figure 3.1.

BDNF$^{2L/2LCk\text{-}cre}$ mutant mice exhibit increased intake of palatable high-fat food. ***A***, Caloric intake of wild-type (WT) and BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant (CM) mice that were fed SC or palatable HFF *ad libitum* (n = 10; *p < 0.0001). ***B***, Caloric intake of high-fat chow of wild-type and BDNF$^{2L/2LCk\text{-}cre}$ mutant mice during a 1 h restricted period over 3 consecutive days (n = 10; *p = 0.0001; **p < 0.0001).

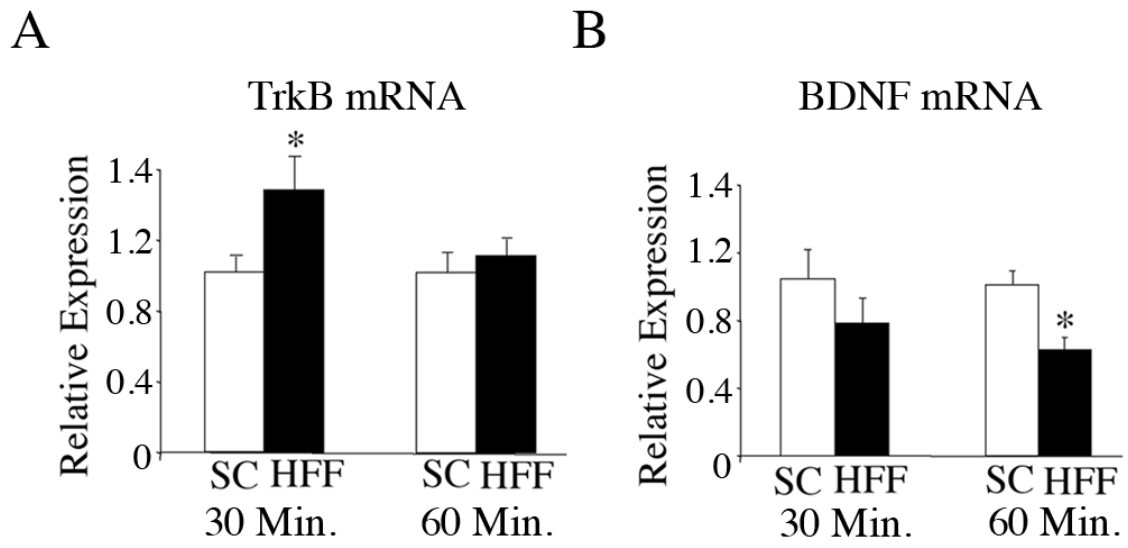


Figure 3.2.

Intake of palatable high-fat food alters levels of BDNF and TrkBmRNAin the VTA of wild-type mice. ***A***, Expression levels of TrkB mRNA in the ventral tegmental area of wild-type mice fed SC or HFF chow for 30 or 60 min as measured by quantitative RT-PCR analysis (n = 5; *p = 0.04). ***B***, Relative expression of BDNF mRNA in the ventral tegmental area of wild-type mice fed standard or high-fat chow for 30 or 60 min (n = 5; *p = 0.004). Data are expressed as fold difference of HFF-fed relative to SC-fed wild-type animals. The mean value for SC-fed mice was set at 1. p values were calculated based on $2^{\Delta\Delta Ct}$ values for each sample.

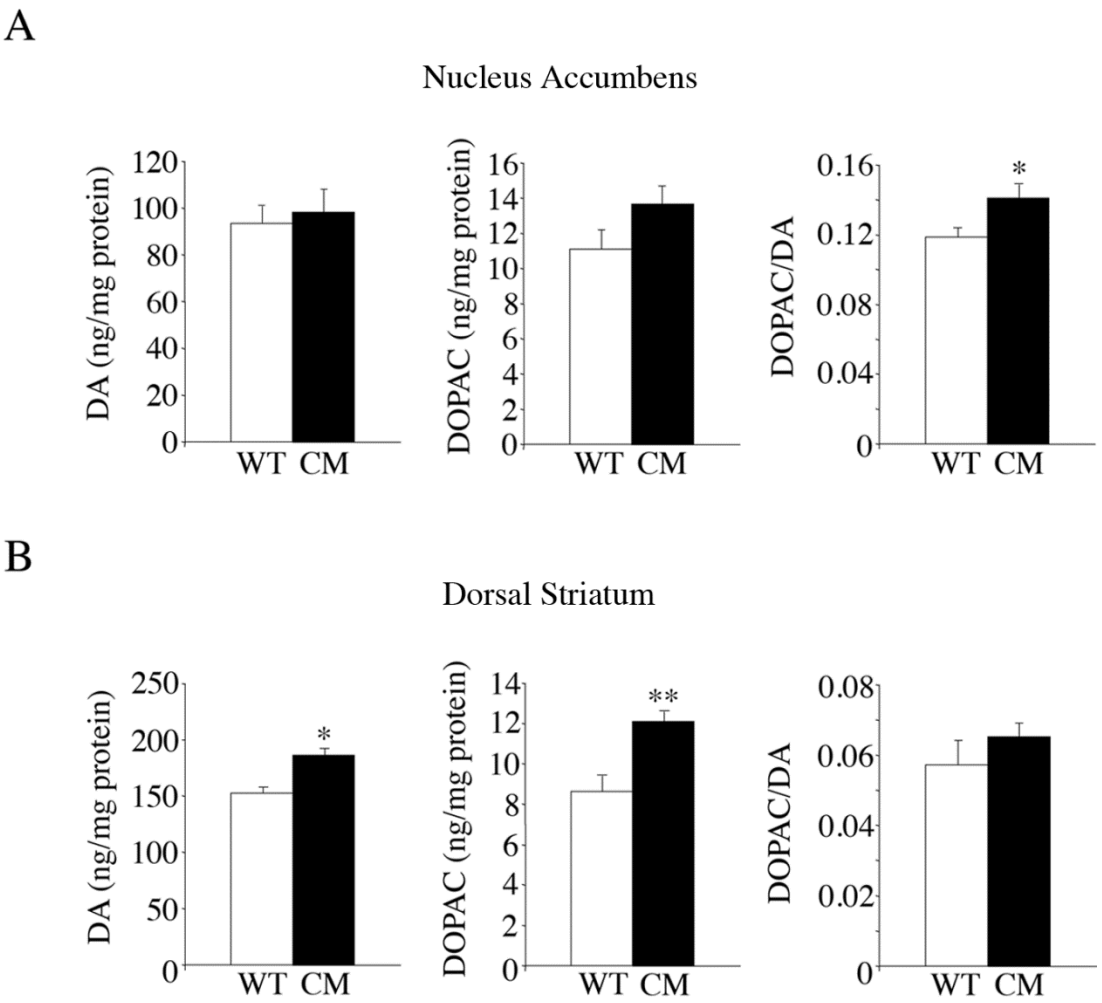


Figure 3.3.

Dopamine content in nucleus accumbens and dorsal striatum of BDNF$^{2L/2LCk\text{-}cre}$ mutant mice. ***A***, HPLC measurements of DA and DOPAC content and DOPAC/DA ratios in the nucleus accumbens of wild-type control (WT) and BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant (CM) mice (n = 5; *p = 0.02). ***B***, Dopamine and DOPAC content and DOPAC/DA ratios in the dorsal striatum of wild-type control and BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant mice (n = 5; *p = 0.002; **p = 0.007).

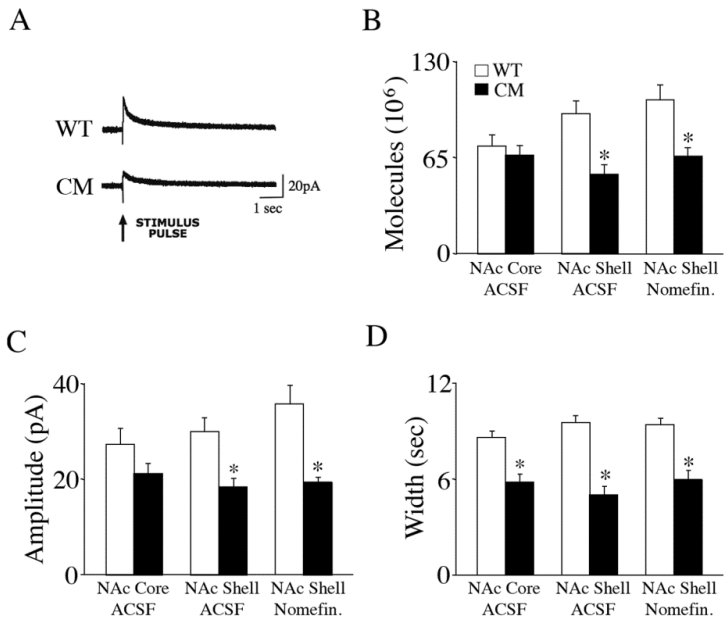


Figure 3.4.

Evoked release of dopamine in the nucleus accumbens shell is impaired in BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant mice. ***A***, Representative amperometric traces of electrical stimulation-evoked dopamine release in acute coronal accumbens slices of wild-type (WT) and BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant (CM) mice. Stimulation electrodes and carbon fiber recording microelectrodes were positioned in the shell region of the nucleus accumbens, which receives the majority of the dopaminergic projections from the VTA. Data were acquired at 50 kHz and digitally postfiltered at 1 kHz. Arrow points to onset of electrical single pulse (2 ms of 0.5 mA). ***B***, Mean evoked dopamine molecules in the nucleus accumbens core (NAc Core) and shell (NAc Shell) in brain slices from wild-type and BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant mice in ACSF and ACSF with the dopamine reuptake inhibitor nomifensine (Nomifen.). $*p < 0.01$. ***C***, Evoked dopamine signal amplitude in the NAc core and shell of wild-type and BDNF$^{2L/2LCk\text{-}cre}$ mutant mice. $*p < 0.01$. ***D***, Evoked dopamine signal width in the NAc core and shell of wild-type and BDNF$^{2L/2LCk\text{-}cre}$ mutant mice. $*p < 0.01$.

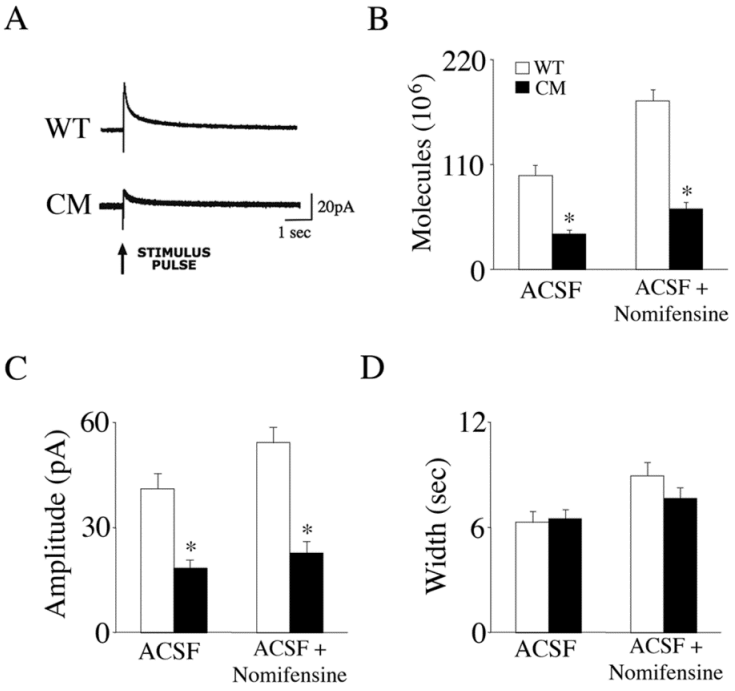


Figure 3.5.

BDNF$^{2L/2LCk\text{-}cre}$ conditional mutants exhibit deficits in evoked release of dopamine in the dorsal striatum. ***A***, Representative amperometric traces of electrical stimulation-evoked dopamine release in acute coronal striatal slices from wild-type (WT) and BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant (CM) mice. Stimulation electrodes and carbon fiber recording microelectrodes were positioned in the medial– dorsal region of the striatum. Data were acquired at 50 kHz and digitally postfiltered at 1 kHz. The arrow points to onset of electrical single pulse (2 ms of 0.5 mA). ***B***, Mean evoked dopamine molecules in the dorsal striatum of wild-type and BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant mice in ACSF and ACSF with the dopamine reuptake inhibitor nomifensine. *$p < 0.01$. ***C***, Evoked dopamine signal amplitude in the dorsal striatum of wild-type and BDNF$^{2L/2LCk\text{-}cre}$ mutant mice. *$p < 0.01$. ***D***, Evoked dopamine signal width in the dorsal striatum of wild-type and BDNF$^{2L/2LCk\text{-}cre}$ mutant mice. *$p < 0.01$.

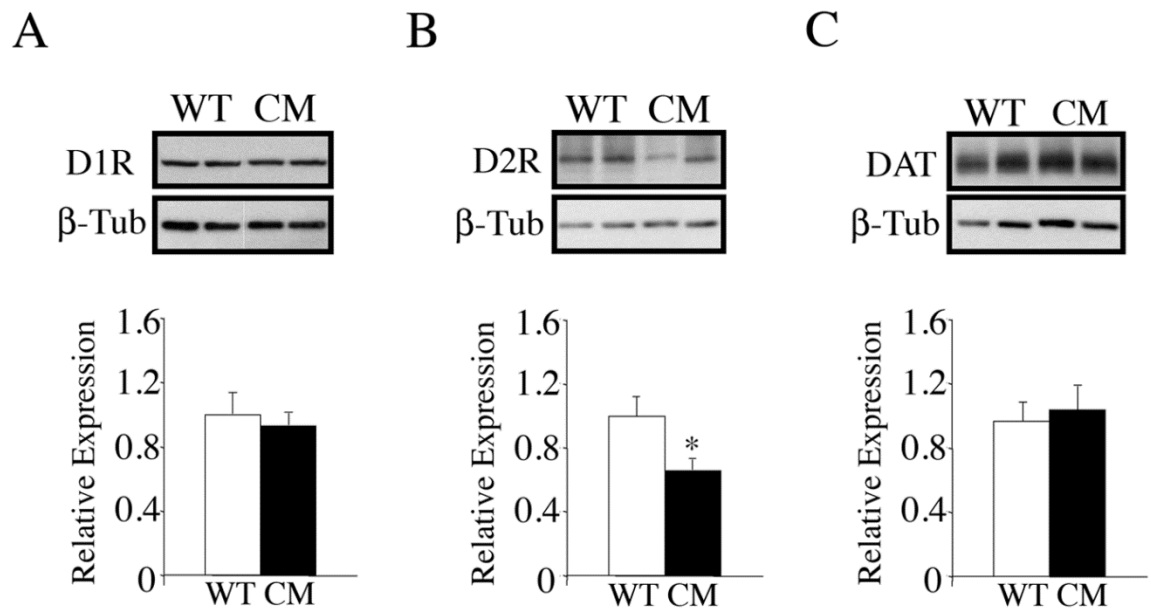


Figure 3.6.

Expression of D2 receptor protein is reduced in the dorsal striatum of BDNF$^{2L/2LCk\text{ -cre}}$ conditional mutants. ***A***, Representative Western blot and densitometry analysis measuring D1R protein content in the dorsal striatum of wild-type (WT) and BDNF$^{2L/2LCk\text{-cre}}$ conditional mutant (CM) mice (n = 5). Data are expressed as expression of BDNF mutant mice relative to wild-type controls. ***B***, Expression of D2R protein in the dorsal striatum of wild-type and BDNF conditional mutant mice (n = 5; p = 0.03). ***C***, Expression of DAT in the dorsal striatum of wild-type and BDNF conditional mutant mice (n = 5).

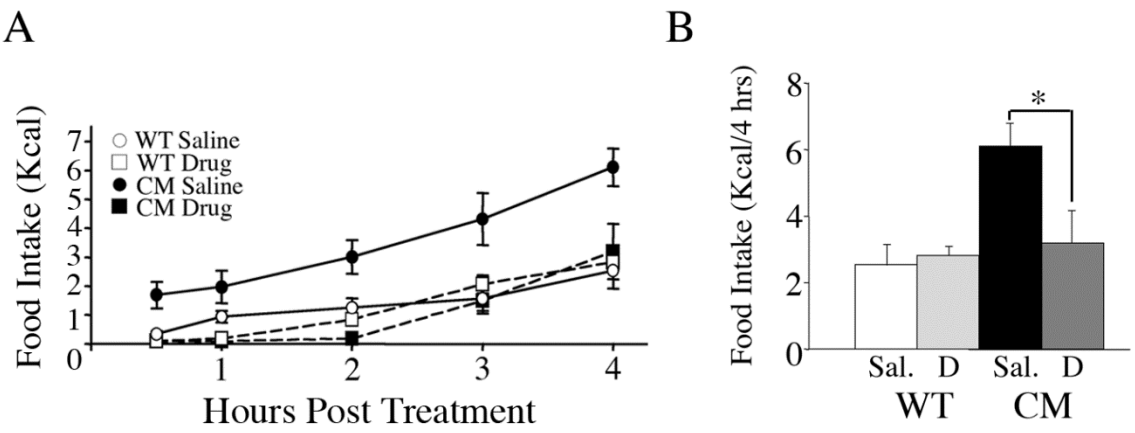


Figure 3.7.

Peripheral administration of the D1 receptor selective agonist SKF-38393 reduced consumption of palatable high-fat food of BDNF$^{2L/2LCk\text{-}cre}$ mutant mice. ***A***, Time course for HFF caloric intake of fed wild-type (WT) and BDNF$^{2L/2LCk\text{-}cre}$ conditional mutant (CM) mice following peripheral administration of saline vehicle or SKF-38393 drug (*n* = 5 per group). ***B***, Four-hour cumulative HFF intake of fed wild-type and BDNF mutant mice following saline vehicle (Sal.) or SKF-38393 drug (D) treatment. **p* = 0.04, *n* = 5 per group.

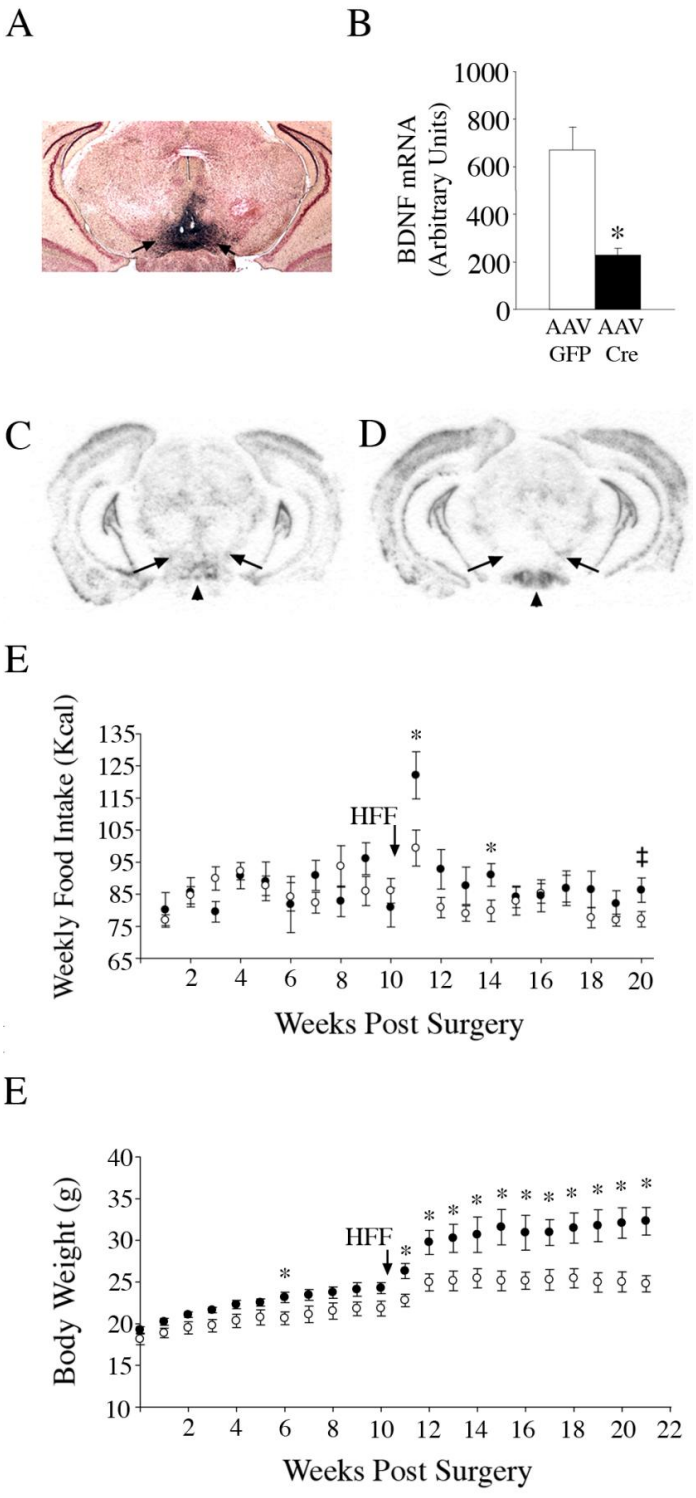
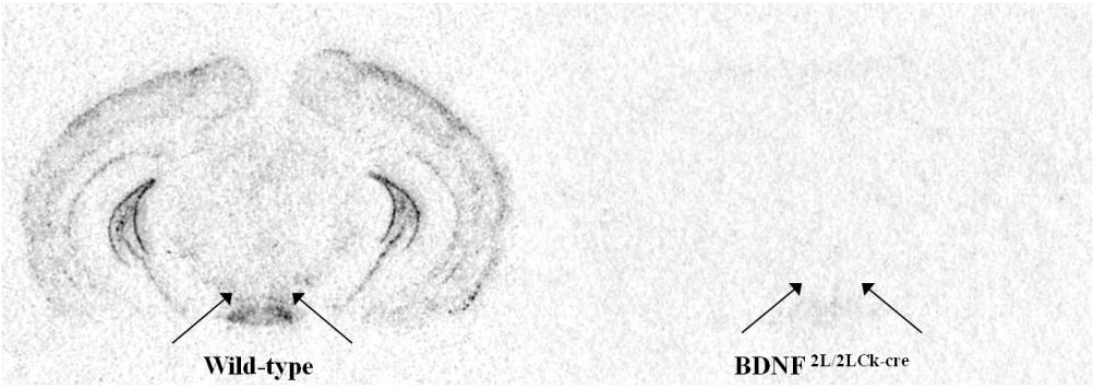


Figure 3.8.

Selective targeting of *Bdnf* in the VTA results in increased intake of palatable HFF and higher body weights.

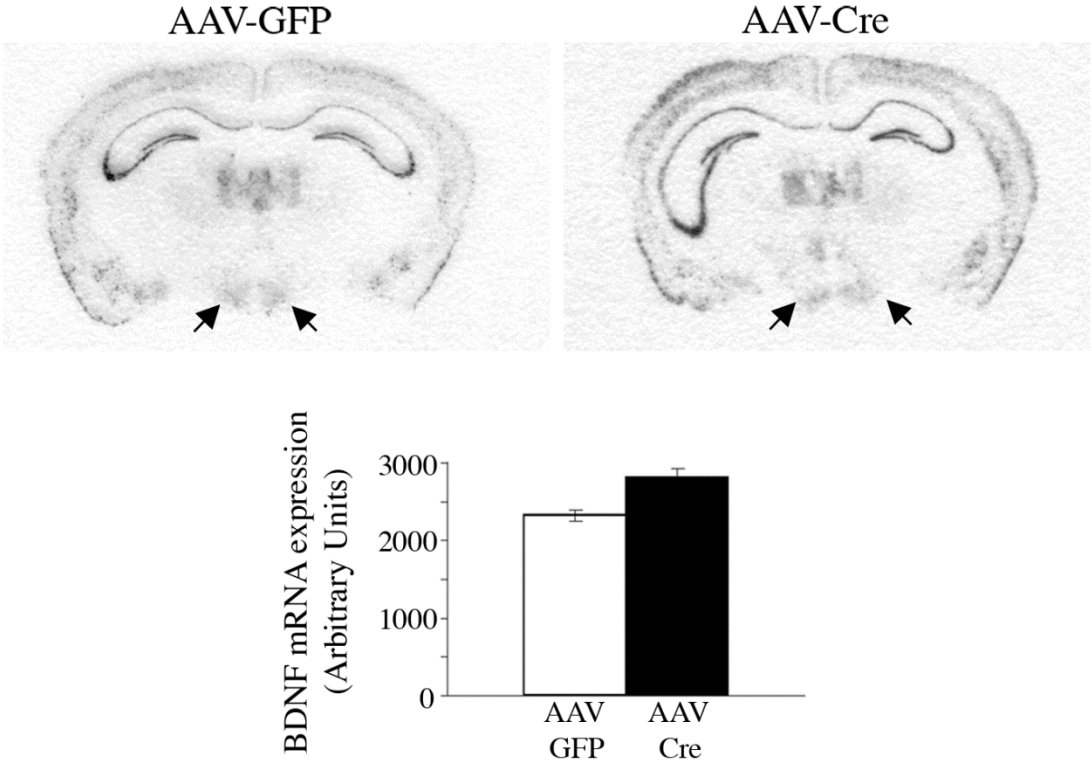
Figure 3.8.

Selective targeting of *Bdnf* in the VTA results in increased intake of palatable HFF and higher body weights. ***A***, Representative coronal brain section from a Rosa-β-gal reporter mouse that was delivered AAV2/1-Cre to the VTA. ***B***, Densitometry of BDNF mRNA signal detected by *in situ* hybridization analysis in AAV2/1-GFP (n = 6) and AAV2/1-Cre (n = 5) injected mice. *p = 0.004. ***C***, ***D***, *In situ* hybridization analysis of BDNF mRNA expression in coronal brain sections from floxed *Bdnf* mice injected with AAV2/1-GFP (***C***) or AAV2/1-Cre (***D***) in the VTA. Arrows indicate the VTA and arrowhead indicates the mammillary nucleus. ***E***, Weekly caloric intake of AAV2/1-GFP (open circles, n = 6) and AAV2/1-Cre (closed circles, n = 5) injected mice under standard and high-fat chow conditions (*$p < 0.05$; ‡, $p = 0.06$). Arrow indicates time point when mice were switched from a standard to a high-fat chow diet. ***F***, Body weights of AAV2/1 GFP (open circles, n = 6) and AAV2/1-Cre (closed circles, n = 5) injected mice over 21 weeks following stereotaxic surgery and under standard and high-fat chow conditions (*$p = 0.05$). Arrow indicates time point when mice were switched from a standard to a high-fat chow diet.



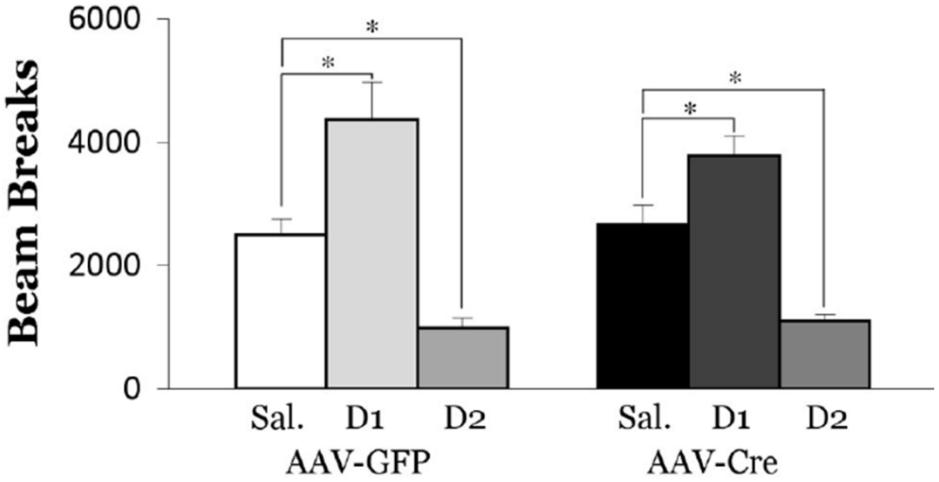
Supplemental Figure 3.1.

In situ hybridization analysis of BDNF mRNA expression in coronal brain sections from wild-type (A) and BDNF$^{2L/2LCk\text{-}Cre}$ (B). Arrows indicate the VTA.



Supplemental Figure 3.2.

In situ hybridization analysis of BDNF mRNA expression in coronal brain sections from floxed *Bdnf* mice injected with AAV2/1-GFP or AAV2/1-Cre in the VTA (top panels) and densitometry of BDNF mRNA signal in the ventromedial hypothalamus of AAV2/1-GFP or AAV2/1-Cre-injected mice. There were no significant differences. Arrows indicate the ventromedial hypothalamus.



Supplemental Figure 3.3.

Locomotor Activity of VTA-specific BDNF mutant mice. Measurements of locomotor activity of AAV2/1-GFP and AAV2/1-Cre-injected mice in their home cages 3 hours following a single injection of saline (Sal.), SKF-81297 (D1) or Quinpirole (D2). *, $P < 0.05$.

Chapter 4:

Discussion

Overview

The studies I have performed and describe here provide novel, mechanistic insight into the anorexigenic effects of BDNF in homeostatic and hedonic feeding circuits in the brain. Chapter 2 of this thesis describes studies identifying α2δ-1 as a novel, appetite-regulating factor mediating the satiety effects of BDNF in the VMH, a region critical in controlling energy homeostasis. Whereas inhibition of α2δ-1 in wild type VMH promoted hyperphagia and weight gain, I demonstrated that rescue of α2δ-1 in the VMH of $BDNF^{2L/2LCk\text{-}cre}$ mice was sufficient to ameliorate obesity as well as key aspects of their metabolic syndrome. Importantly, I also presented electrophysiological, transcriptional, and immunohistochemical evidence suggesting that the satiety effects of α2δ-1 may be mediated by interactions with TSP-3, one of its endogenous ligands known to promote excitatory synaptogenesis in the CNS. These findings identify a previously unrecognized role for α2δ-1, help to define the mechanistic action of BDNF signaling in homeostatic control of appetite and offer insight on new strategies for the treatment and prevention of obesity.

Chapter 3 of this thesis outlines investigations that uncovered an essential role for BDNF in hedonic feeding through positive regulation of the mesolimbic dopamine pathway. I showed that central BDNF depletion in mice significantly decreased dopamine secretion in the NAc and dorsal striatum. Moreover, $BDNF^{2L/2LCk\text{-}cre}$ mice display excessive intake of palatable food which is normalized by D1 receptor stimulation. Notably, VTA-specific deletion of *Bdnf* resulted in increased feeding and body weight when given a palatable high-fat diet. Altogether, these data significantly advance our understanding of the multiple and neural substrate specific roles for BDNF in the regulation of appetitive behaviors wherein BDNF dysfunction contributes to the pathogenesis of abnormal food intake and obesity.

The Actions of BDNF in Energy Homeostasis

α2δ-1 is a novel satiety factor acting downstream of BDNF

Until recently, the primary function attributed to α2δ-1 was to mediate calcium channel activity by enhancing trafficking of the Ca_V complex to the plasma membrane (Dooley et al., 2007). Accordingly, chronic application of Gabapentin (GP; Neurotonin) and Pregabalin (PGB; Lyrica), which bind with high affinity to α2δ-1 to pharmacologically inhibit membrane trafficking, decrease surface expression of the Ca_V complex and decrease Ca^{2+} currents (Heblich et al., 2008; Hendrich et al., 2008). This is thought to be the mechanism mediating the anti-allodynic and anti-epileptic actions of gabapentinoid drugs (Field et al., 2006). Of note, the use of Neurotonin or Lyrica have been reported to induce dramatic weight gain in patients ((DeToledo et al., 1997; Hoppe et al., 2008) through unknown mechanisms. Here, I present evidence that α2δ-1 is a novel satiety factor acting in the VMH. These findings help explain the weight gain associated with gabapentinoid drug treatment in patients afflicted with pain and seizure disorders (DeToledo et al., 1997; Hoppe et al., 2008), an important step toward developing alternative therapies minimizing or preventing this negative side effect. Interestingly, while the viral-mediated rescue of α2δ-1 in the VMH of $BDNF^{2L/2LCk\text{-}cre}$ mice significantly reduced their hyperphagia and obesity, it did not completely abolish these alterations. Several possibilities explain this outcome.

First, although post-hoc analysis using quantitative RT-PCR revealed that α2δ-1 transcript was increased by an average of 50% in the VMH of $BDNF^{2L/2LCk\text{-}cre}$ mice administered AAV- α2δ-1, this may not translate to normalized, WT levels of α2δ-1 protein at the cell surface. Thus, an incomplete rescue of the WT phenotype might reflect an incomplete rescue of α2δ-1 protein at the cell surface. Consistent with this possibility, food

intake is negatively correlated with percent rescue of α2δ-1 in the VMH of BDNF$^{2L/2LCk\text{-}cre}$ mice (R^2 = .98). This suggests that levels of α2δ-1 at the cell surface might dose-dependently influence food intake behavior leading to obesity. Ongoing studies in the lab aim to answer these questions directly by using a biotinylation assay to examine the level of α2δ-1 protein at the cell-surface in AAV- α2δ-1 rescued BDNF$^{2L/2LCk\text{-}cre}$ mice.

Another possibility explaining incomplete recovery of wild type feeding in BDNF$^{2L/2LCk\text{-}cre}$ mice is that while the α2δ-1 rescue was selective to the VMH, the satiety effects of α2δ-1 might not restricted to the VMH. α2δ-1 is highly expressed in the PVN and DVC (Taylor & Garrido, 2008), energy balance-regulating regions where BDNF signals for satiety (Bariohay et al., 2005; Toriya et al., 2010; C. Wang, Bomberg, Billington, Levine, & Kotz, 2007a). α2δ-1 is also moderately expressed in the VTA, where I demonstrated (in Chapter 3) that BDNF is critical for the control of palatable food intake (Cordeira, Frank, Sena-Esteves, Pothos, & Rios, 2010). It was recently shown that α2δ-1 levels are increased significantly in the NAc and the frontal cortex of mice with repeated methamphetamine treatment. The elevation of α2δ-1 as well as the behavioral sensitivity to methamphetamine, including increased locomotor activity and place preference, was dose-dependently suppressed with co-administration of gabapentin (Kurokawa, Shibasaki, Mizuno, & Ohkuma, 2011). These results suggest that α2δ-1 may also serve a functional role in the mesolimbic dopamine system.

While these possibilities remain to be explored, it is intriguing to hypothesize that α2δ-1 dysfunction in other appetite regulating centers of the brains in BDNF$^{2L/2LCk\text{-}cre}$ mice, which are uninfluenced by viral rescue of α2δ-1 in the VMH, might continue to support an obesity phenotype. If α2δ-1 serves to mediate satiety by acting in multiple brain regions,

then complete rescue of the wild type phenotype in BDNF$^{2L/2LCk\text{-}cre}$ mice would necessitate rescue in each of these regions, not only the VMH. Future studies will be directed at exploring the potential satiety effects α2δ-1 in these alternate regions. Nonetheless, as shown here, direct modulation of α2δ-1 in the VMH is sufficient to alter feeding behavior and bodyweight.

Structural motifs, transcriptional alterations, and altered trafficking of α2δ-1

I showed that levels of α2δ-1 transcript in the VMH of BDNF$^{2L/2LCk\text{-}cre}$ mice were decreased in the transcriptional profiling experiments but not in the RT-PCR analysis. Furthermore, total protein levels of α2δ-1 in the VMH of BDNF mutants were normal. These discrepancies are unlikely to be reconciled as a simple false-positive identified by the gene array. In support of this, α2δ-1 transcript was identified as downregulated in the array by three independent probe sets in VMH samples from two independent strains of mice: BDNF$^{2L/2LCk\text{-}cre}$ mice and VMH-specific *Bdnf* knockout mice. Importantly, the α2δ-1 protein analysis showing decreased cell surface expression in BDNF mutants and the efficacy of the α2δ-1 functional rescue further demonstrated dysfunction in BDNF$^{2L/2LCk\text{-}cre}$ mice. Because α2δ-1 is alternatively spliced (A. Davies et al., 2007), it is possible that the gene array data reflect alterations in RNA splicing in BDNF mutants that ultimately impact cell surface protein expression but not total content of α2δ-1. Consistent with this idea, introduction of α2δ-1b cDNA using a viral vector, which bypasses RNA processing, was capable of attenuating the hyperphagia and metabolic alterations associated with the α2δ-1 deficit in BDNF mutant mice. Furthermore, a precedent for this possibility is supported by recent

evidence showing that BDNF-induced alternative splicing is required to produce a functionally active Xbp1 protein in the mouse brain (Hayashi et al., 2007).

Alternative splicing is a common mechanism which produces a diversity of protein species from the same primary transcript; which often exhibit differential expression patterns, biochemical properties, and cellular localization. There are multiple structural elements and domains within α2δ-1, including VWA, MIDAS, GPI anchors, and TM domains that are vital for proper trafficking, localization, and function of α2δ-1 and α1. Thus, alternative splicing of the α2δ-1 transcript, which may influence the structural elements directing the protein to the cell surface, would constitute a potential mechanism through which α2δ-1 is differentially trafficked to the membrane.

Alternative splicing produces at least 5 isoforms of α2δ-1_{A-E} which follow a tissue specific pattern of expression, where α2δ-1_B is specific to the brain. These splice variants have not been fully characterized and the functional significance of their expression pattern has yet to be established (Angelotti & Hofmann, 1996). Of note, however, four of the natural variations of the transcript are located in a region of α2δ-1 transcript immediately adjacent to and partially overlapping the VWA domain. Because mutations to the VWA domain prevent membrane trafficking of α2δ-1 and α1 , known or as yet unrecognized isoforms with splicing alterations and peptide variations near this region may alter the affinity with which ligands are capable of binding α2δ-1 and/or decrease membrane trafficking.

Splice alterations which produce peptide variations may also interfere with the ability of α2δ-1 to perform the conformational shift suggested to be important for the activation of trafficking. Changes in protein folding may serve to expose or mask signaling motifs

sufficient to promote membrane trafficking. In support of this possibility, although specific retention motifs have not yet been identified, Cornet et al. demonstrate that ER retention of α1 is suppressed by the appropriate folding of $Ca_v\alpha1$ in order to mask several internal ER-retention sites. The interaction with $Ca_V\beta$, another subunit facilitating Ca_V trafficking, is also critical to suppress ER-retention signals in $Ca_v\alpha1$ (Cornet et al., 2002). Moreover, the trafficking and surface expression of NMDA receptor subunits is also mediated by alternative splicing which either exposes or masks an RXR retention motif in the C-terminus (Scott, Blanpied, Swanson, Zhang, & Ehlers, 2001). Because BDNF has been shown to regulate the trafficking of NMDA receptors in hippocampal neurons (Caldeira et al., 2007), it is interesting to speculate whether BDNF-mediated alternative splicing regulates α2δ-1 trafficking using similar mechanisms. Future studies will address these possibilities. Nevertheless, these examples reaffirm the importance of transcript splicing, peptide folding, and interactions which determine protein localization and function. It moreover highlights potential sites for dysfunction leading to altered α2δ-1 trafficking and localization.

Calcium channel-independent functions for α2δ-1

VMH neurons in $BDNF^{2L/2LCk\text{-}cre}$ mice display a significant decrease in α2δ-1 surface expression yet calcium channel currents are unaltered. This suggests a role for α2δ-1 in appetite control that is independent of calcium channel function. Interestingly, the VWA domain of α2δ-1 participates in protein-protein interactions with thrombospondins, a family of extracellular matrix proteins (Eroglu et al., 2009). TSPs bind VWA to mediate excitatory synaptic plasticity in neurons. Because TSP/α2δ-1-induced synaptogenesis occurs in the

presence of calcium channel blockade, α2δ-1 appears to function independently from Ca_V in this context (Eroglu et al., 2009).

Consistent with Ca_V-independent roles, α2δ-1 is not always found in physical association with the α1 subunit. For example, α2δ-1 and α1 mRNA (Morton & Froehner, 1989; Varadi, Orlowski, & Schwartz, 1989) and protein (Morton & Froehner, 1989) are expressed in temporally and spatially distinct patterns during muscle cell development. While α1 subunits cluster in the center of developing myotubes, α2δ-1 shows strong immunolabeling at the ends, where α1 labeling is weak or absent (Garcia, Nabhani, & Garcia, 2008). Notably, siRNA mediated knockdown of α2δ-1in myotubes has very little effect on calcium channel currents. Cell adhesion processes requiring extracellular signaling via VWA, however, were dramatically inhibited (Garcia et al., 2008). Taken together, these data are consistent with the possibility that α2δ-1 may exist at the plasma membrane in the absence of α1, and that α2δ-1 may independently function in novel ways beyond the traditional role of calcium channel trafficking. This is an intriguing idea, since biologically it would define a separation of functions for α2δ-1. Regulation of α2δ-1/VWA signaling to promote synaptogenesis (and potentially food intake) would not necessarily need to be at the expense of Ca^{2+} signaling, which is a cellular necessity for survival.

Thrombospondins are endogenous α2δ-1 ligands that may regulate food intake

Evidence by Eroglu et al., imply that at least some novel roles for α2δ-1 involve extracellular signaling via VWA domains. In particular, they demonstrate that the EGF-like repeats present in all 5 isotypes of TSPs bind to the VWA domain of α2δ-1 to promote excitatory synaptogenesis in the CNS (Christopherson et al., 2005; Eroglu et al., 2009).

Interestingly, we and others present evidence linking TSPs to food intake and bodyweight regulation, suggesting that TSPs may impact energy balance through interactions with α2δ-1. In wild type mice, for example, I have shown that TSP-3 expression is dramatically decreased in the DMH under fasted conditions. In hyperphagic and obese $BDNF^{2L/2LCk\text{-}cre}$ mice, TSP-3 is significantly increased in the DMH, suggesting a potential homeostatic, compensatory response to decreased α2δ-1 receptor. Moreover, I observed TSP-3^{+} neuronal fibers traveling between the VMH and DMH, suggesting that connectivity between these nuclei may play an important function in feeding.

Evidence from other research groups demonstrates that TSP-1, produced by visceral adipose tissue, is highly expressed in obese, insulin-resistant subjects (Varma et al., 2008). In addition, female TSP-2 null mice are 30% heavier than wild type controls and overeat standard chow (Shitaye, Terkhorn, Combs, & Hankenson, 2010). TSP-3 null mice are also transiently heavier than controls but their weights normalize with age perhaps due to compensatory effects of other TSP's (Hankenson, Sweetwyne, Shitaye, & Posey, 2010; Posey et al., 2008). Taken together, the cumulative data suggest that the satiety effects of α2δ-1, mediated by BDNF, might be related to interactions with TSPs. Furthermore, we propose that TSP-3 from cells in the DMH might participate in appetite control by serving as a ligand for α2δ-1 in cells in the VMH to facilitate synaptic connectivity between these two cell populations.

A model of α2δ-1 and TSP-3 interactions in the hypothalamus mediating satiety

Hypothalamic circuits are dynamically reorganized according to energy status and metabolic signals. For example, the strength of excitatory inputs from the medial VMH onto POMC neurons in the Arc is diminished by fasting (Sternson et al., 2005). Under these low energy conditions, the number of excitatory inputs onto orexigenic NPY/AgrP are concomitantly elevated (Y. Yang, Atasoy, Su, & Sternson, 2011). Hyperphagic and obese (*ob/ob*) mice display a similar synaptic profile to food-deprived mice; higher synaptic tone on to NPY neurons. Leptin treatment in *ob/ob* mice, however, rapidly normalizes the synaptic profile by increasing excitatory input onto POMC neurons (Pinto et al., 2004). Similarly, administration of ghrelin (Pinto et al., 2004) or estradiol (Parducz, Zsarnovszky, Naftolin, & Horvath, 2003) in WT mice induces synaptic reorganization in arcuate neurons in a manner consistent with their respective orexigenic and anorexigenic effects. The collective data suggest that dynamic structural changes in the hypothalamus are central to regulating feeding behavior.

Considering the well ascribed roles of BDNF facilitating synaptic plasticity in the brain (Lu & Figurov, 1997), it is reasonable to speculate that it might participate in synaptic remodeling of feeding circuits. Consistent with this idea, BDNF administration to the VMH does not significantly decrease feeding until 4-24 hrs and 48 hrs following treatment (C. Wang, Bomberg, Billington, Levine, & Kotz, 2007a). This is contrast to the PVN, where BDNF administration rapidly decreases food intake within the first 1-2 hours following treatment (C. Wang, Bomberg, Billington, Levine, & Kotz et al., 2007b). This delayed effect of BDNF in the VMH supports a time frame during which it may trigger synaptic remodeling, via regulation of α2δ-1 and its interactions with TSP-3 (Fig 4.1).

Our model proposes that TSP-3+ neurons interconnect the DMH and VMH and interact with post-synaptic α2δ-1 in cells of the VMH. By interacting with post-synaptic α2δ-1 to promote synaptogenesis, TSP-3 may regulate excitatory synaptic tone onto VMH neurons. Because α2δ-1 contains a small cytoplasmic domain, intracellular signaling supporting synaptogenesis might be mediated through an undetermined binding partner in a Ca_V-independent fashion. Nevertheless, enhanced excitatory tone on VMH neurons, which is critical to maintenance of anorexigenic output, represents a mechanism that would promote satiety. According to this model (Fig 4.1), decreased levels of TSP-3 in the DMH during fasting would decrease interaction with α2δ-1 in the VMH. This would subsequently decrease excitatory input onto VMH neurons and decrease their anorexigenic output, ultimately promoting feeding. Reduced α2δ-1 at the cell surface, which I observed in the VMH of BDNF depleted mice, would decrease binding sites for TSP-3 and decrease anorexigenic output. This is consistent with the behavioral hyperphagia observed in $BDNF^{2L/2LCk\text{-}cre}$ mice. Thus, by regulating surface expression of α2δ-1, BDNF might mediate TSP-3-induced synaptic connectivity between hypothalamic nuclei to control food intake.

Because α2δ-1 is also located at pre-synaptic terminals (Taylor & Garrido, 2008), it is alternatively possible that pre-synaptic α2δ-1-containing fibers from the VMH interact with neuronal or glial-derived TSP-3 in the DMH to increase the excitatory drive of anorexigenic neurons within the DMH (Fig 4.1B-C). For example, since the DMH responds to direct administration of CCK, a gut-derived satiety factor, as well as MC4R agonists by reducing feeding (Blevins et al., 2000; P. Chen et al., 2004), it is possible that local excitatory synaptogenesis could promote satiety by increasing the anorexigenic output of these neurons.

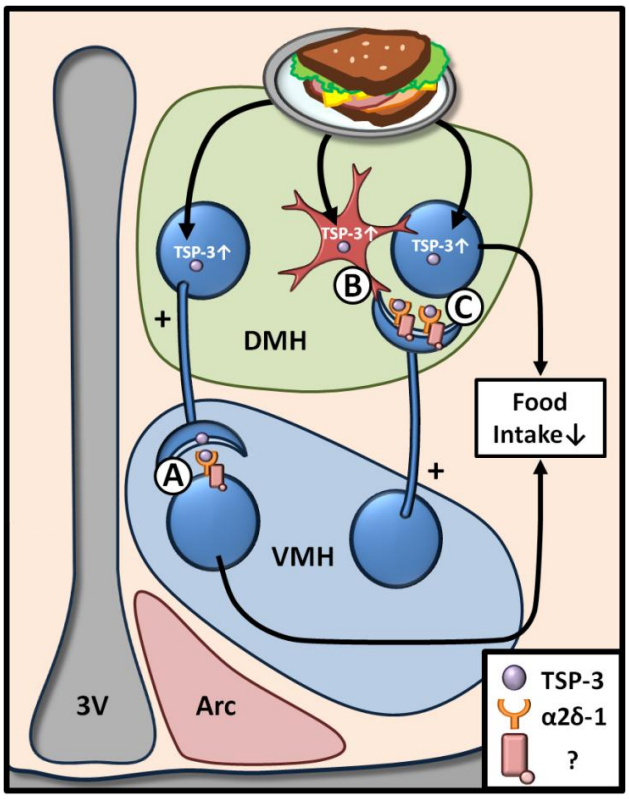


Figure 4.1

A model of α2δ-1 and TSP-3 interactions in the hypothalamus mediating satiety.

In the fed state, (A) elevated levels of TSP-3 in neuronal fibers originating in the DMH interact with post-synaptic α2δ-1 receptors in anorexigenic VMH cells. TSP-3-mediated activation of α2δ-1 initiates intracellular signaling cascades that promote synaptogenesis and increased excitatory input onto VMH cells, increasing the anorexigenic tone. Because α2δ-1 contains a small cytoplasmic domain, intracellular signaling might be mediated through an undetermined binding partner in a calcium-independent fashion. α2δ-1 could also act pre-synaptically in VMH fibers projecting to the DMH, serving as a receptor for TSP-3 produced by astrocytes (B) or neurons (C) to mediate excitatory synaptogenesis onto anorexigenic neurons in the DMH. Arc, arcuate nucleus; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus; 3V, third ventricle.

The Actions of BDNF in Hedonic Feeding Control

Decreased dopamine transmission mediates hedonic feeding in BDNF$^{2L/2LCk\text{-}cre}$ mice

In Chapter 3, I demonstrated an intimate capacity for BDNF to regulate hedonic feeding by positive modulation of the mesolimbic dopamine system. BDNF$^{2L/2LCk\text{-}cre}$ mice with limited access to palatable, high-fat chow exhibited dramatic binge eating behavior; a phenotype which appeared to be the result of defective DA transmission. In support of this, amperometry in acute brain slices from BDNF$^{2L/2LCk\text{-}cre}$ mice revealed marked deficits in evoked DA secretion. The decrease in evoked secretion persisted in the presence of nomifensine, a DAT reuptake inhibitor, indicating that the deficit was due to decreased secretion and not increased reuptake of dopamine. Total basal levels were also normal, suggesting DA synthesis was uninfluenced by BDNF depletion. Interestingly, evoked secretion was decreased in the NAc shell but not the NAc core. These data are consistent with work demonstrating that VTA-derived DA is preferentially released into NAc shell, as opposed to the core, following palatable food consumption (Bassareo & Di Chiara, 1999). Moreover, local inhibition of the NAc shell via application of muscimol, a $GABA_A$ agonist, elicits intense hyperphagia in rats (Baldo et al., 2005; Stratford et al., 1998).

Defective DA secretion in the NAc has been linked to hedonic feeding and obesity. For example, evoked DA release is severely attenuated in the NAc of diet-induced obese and obesity-prone rats, as well as *ob/ob* mice (Geiger et al., 2008; Geiger et al., 2009; Hommel et al., 2006). Importantly, *ob/ob* mice reduce food intake and body weight when treated with DA agonists (Bina & Cincotta, 2000). These results are comparable to the deficient DA secretion we observed in the NAc of BDNF$^{2L/2LCk\text{-}cre}$ mice. Moreover, when BDNF$^{2L/2LCk\text{-}cre}$ mice were similarly treated with the selective D1 receptor agonist, they dramatically reduced

consumption of palatable, high-fat food. These studies suggest that defective dopamine transmission in the NAc underlie the hedonic feeding triggered by BDNF depletion in $BDNF^{2L/2LCk\text{-}cre}$ mice.

We observed additional deficits in dopamine secretion in the dorsal striatum of BDNF mutants which also persisted in the presence of nomifensine. Interestingly, increased DA content was measured in the mutant dorsal striatum, perhaps indicating a compensatory increase in synthesis due to deficits in release. A recent publication independently confirmed our findings. In BDNF heterozygous mice, decreased evoked secretion with elevated basal levels of DA was also observed in the dorsal striatum (Bosse et al., 2012). Consistent with studies demonstrating the ability for BDNF to potentiate the release of DA from rat striatal slices and mesenphalic neurons (Blochl & Sirrenberg, 1996; Goggi et al., 2003), Bosse et al. demonstrated that exogenous BDNF application was able to rescue DA release in BDNF heterozygous mice.

DA transmission in the dorsal striatum, which is densely innervated by DA fibers from the substantia nigra, has also been linked to food reward processes. For example, striatal dopamine signaling via D2R is reduced in obese patients (Stice et al., 2008) and the degree of pleasure obtained from palatable food is correlated to the level of DA release in the striatum (D. M. Small et al., 2003). There are, however, distinctions between hedonic feeding and DA signaling in the NAc and the dorsal striatum which I believe are best depicted by studies involving DA deficient mice. These mice exhibit a decrease in food intake and, without intervention, fail to eat enough to survive beyond 48 hours. Whereas restoration of DA in the NAc rescued consumption of sucrose, palatable diet, and exploratory behavior, DA production in the dorsal striatum restored feeding of both standard and

palatable chow (Szczypka et al., 2001). These results support the possibility that DA release in the ventral and dorsal striatum differentially encodes the reward/motivational value of palatable food. It is interesting to consider, for example, that while the ventral striatum may be responsible for encoding the motivational value of palatable food, the dorsal striatum might be responding to the caloric value of palatable food. The results are also consistent with Berridge's work describing "incentive salience" wherein VTA lesioned animals continue to show behavioral signs of "liking" palatable food (i.e. affecting facial expressions and lip smacking) in the absence of NAc DA release. These animals, however, lack the motivation or "wanting" to pursue the reward, which can be restored by DA signaling in the NAc (Berridge, 2009; Szczypka et al., 1999; Szczypka, Rainey et al., 1999). While we cannot rule out the possibility that deficient DA signaling in the dorsal striatum of BDNF mutants contributes to their excessive feeding, it is nonetheless clear from our studies that the control of hedonic feeding requires BDNF in the VTA, which sends projections to the NAc.

The VTA is an essential source of BDNF mediating hedonic feeding

The VTA is a major source of BDNF from where it can be released locally and act on TrkB receptors to enhance dopamine neuron excitability in the VTA (Guillin et al., 2001; Seroogy et al., 1994). I found that BDNF expression in the VTA is critical for the regulation of palatable, high-fat food intake. Viral-mediated depletion of *Bdnf* in the VTA significantly increased palatable, high-fat food intake (but not standard chow) leading to obesity (Cordeira et al., 2010). In addition, depletion of *Bdnf* in the PFC had no influence on either standard or palatable chow consumption (my own unpublished data). Although both the PFC and VTA supply BDNF to the mesolimbic DA system, these data indicate the VTA is an essential

source of BDNF mediating hedonic feeding. Further corroborating our results, it was recently shown that RNAi mediated BDNF depletion in the VTA promotes long term weight gain (Fanous, Terwilliger, Hammer, & Nikulina, 2011). Importantly, the diet-specific effects of deleting *Bdnf* in the VTA also suggest that the effects of BDNF on feeding behavior are neural substrate dependent. Whereas BDNF signaling in the VMH is critical for homeostatic feeding mechanisms (Unger et al., 2007; C. Wang, Bomberg, Levine et al., 2007), BDNF signaling in the VTA is essential for the regulation of hedonic feeding.

Potential disease mechanisms contributing to overeating and obesity

Conflicting disease mechanisms have been proposed explaining the mechanisms linking altered dopaminergic transmission to the emergence of eating disorders and obesity. One model suggests that DA systems are involved in associative-reward learning. In this case, heightened DA release upon palatable food consumption overvalues the stimulus, leading to exaggerated eating habits (Dawe et al., 2004). It has alternatively been proposed that hypoactivity within this DA circuitry underlies excessive eating behavior. Specifically, this model proposes that attenuated DA release and/or reduced receptor signaling leads to a reward deficiency syndrome which can be compensated for by pathological eating (Pothos et al., 1998; G. J. Wang et al., 2002). Our results are consistent with the latter model wherein $BDNF^{2L/2LCk\text{-}cre}$ mice display decreased DA secretion. We propose that perturbed BDNF signaling, likely in the VTA, reduces dopaminergic tone in the mesolimibic dopamine pathway leading to reward deficiency and compensatory overeating of palatable food. Additional support for this model comes from mice overexpressing ΔFosB, which display reduced BDNF and molecular changes in the NAc consistent with decreased DA signaling,

including reduced pCREB and DARPP-32. However, six weeks of high-fat diet completely ameliorate these deficits, indicating that hypoactive dopamine signaling can be normalized by overeating (Teegarden et al., 2008).

It is important to consider whether deficits in reward processing precede and contribute to obesity by promoting hedonic feeding, or whether a hypofunctioning dopamine system is a neuro-adaptive response to overconsumption. Significant DA impairment in pre-obese, obesity prone rats supports the possibility that altered dopaminergic transmission is involved in the development of obesity (Geiger et al., 2008). However, animal studies also indicate that chronic excessive intake of palatable chow produces a downregulation of post-synaptic D2 receptors in the striatum (Johnson & Kenny, 2010). Indeed, reduced levels of D2R are observed in the striatum of obese rats (Geiger et al., 2008; Geiger et al., 2009; Thanos et al., 2008) and humans (Stice et al., 2008). In Chapter 3, I showed a significant downregulation of D2R in the dorsal striatum of BDNF mutants. Because alterations in D2R and evoked DA secretion were identified in pre-obese $BDNF^{2L/2LCk\text{-}cre}$ mice, it is likely that hypoactivity of the mesolimbic dopamine circuitry precedes and promotes hedonic feeding leading to obesity, at least in the $BDNF^{2L/2LCk\text{-}cre}$ mouse model.

Mechanisms for decreased DA transmission in $BDNF^{2L/2LCk\text{-}cre}$ mice

As discussed earlier, the VTA is an essential source of BDNF within the mesolimbic DA pathway mediating hedonic feeding regulation. While this represents an important discovery identifying a novel role for BDNF in hedonic feeding, the precise mechanisms through which BDNF in the VTA regulates DA activity and dopamine release remain to be fully elucidated. This is due, in part, to the complexity of the VTA, which contains a

heterogeneous population of neurons. While a majority of neurons here are dopaminergic, distinct GABAergic and glutamatergic neuronal populations have also been identified (Hur & Zaborszky, 2005; Nair-Roberts et al., 2008). They send local collaterals to mediate the activity of DA neurons as well as the release of DA into the NAc. Thus, the activity levels of DA neurons in the VTA, which are associated with reward and motivation to consume palatable food, also depends on activity from excitatory and inhibitory inputs converging on these cells. BDNF may modulate this local circuitry to mediate DA neuron activity. There are several putative mechanisms through which BDNF signaling might regulate DA activity and hedonic feeding.

First, BDNF signaling within DA neurons may directly enhance DA release. Amperometry studies revealed decreased evoked DA secretion in the NAc of $BDNF^{2L/2LCk\text{-}cre}$ mice. Since secretion is evoked by electrical stimulation at the synaptic terminals, mechanisms mediating neuronal excitability at the cell body are not present in these slices and thus, decreased secretion might be due to alterations in neurotransmitter release machinery (Fig. 4.2A). This is supported by reports demonstrating that synaptobrevin and synaptophysin are decreased in BDNF heterozygous mice (Pozzo-Miller et al., 1999) and synapsin mediates the facilitation of neurotransmitter release by BDNF (Jovanovic, Czernik, Fienberg, Greengard, & Sihra, 2000). Moreover, the activity of vesicular monoamine transporter 2 (VMAT2), which acts to transport DA into synaptic vesicles, is significantly decreased in the dorsal striatum of BDNF+/- mice (Boger et al., 2011). Lastly, BDNF-TrkB-PLC-γ signaling to promote intracellular Ca^{2+} release has been shown to directly trigger DA release in amacrine cells (Neal, Cunningham, Lever, Pezet, & Malcangio, 2003). These data

collectively suggest that a lack of BDNF may inhibit the vesicle loading and exocytosis of DA, consistent with decreased DA secretion in the $BDNF^{2L/2LCk\text{-}cre}$ mouse.

Deficits in the neurotransmitter release machinery do not preclude the possibility that there are also problems contributing to the excitability of DA neurons in $BDNF^{2L/2LCk\text{-}cre}$ mice. Thus, another mechanism might involve regulation of GABAergic signaling in the VTA DA neurons by BDNF (Fig 4.2B). Intra-VTA administration of the $GABA_A$ agonist, muscimol, decreases DA release in NAc (Westerink, Kwint, & deVries, 1996), supporting an important role for GABAergic inhibition of DA neurons. BDNF has been shown to reduce $GABA_A$ receptor–mediated inhibition in hippocampal neurons (Tanaka, Saito, & Matsuki, 1997). Furthermore, in neuroendocrine neurons in the PVN, BDNF appears to silence GABA synapses by removing $GABA_A$ receptors using a post-synaptic mechanism (Hewitt & Bains, 2006). These data imply that a loss of BDNF signaling could enhance inhibition and decrease the firing capacity of DA neurons due to lack of $GABA_A$ regulation at the cell surface.

A third candidate mechanism mediating the effects of BDNF might entail regulation of excitatory drive on to VTA DA neurons (Fig 4.2C). Glutamatergic neurons in the VTA also make local synapses onto DA neurons (Dobi et al., 2010), which may serve to enhance the excitability of DA neurons and promote DA release. Indeed, food restriction decreases TrkB levels (representing a scenario of decreased BDNF signaling) in addition to EPSC amplitude in VTA DA neurons, suggesting alterations in glutamate transmission (Pan et al., 2011). BDNF has known roles in enhancing post-synaptic transmission by regulating the expression, trafficking, and phosphorylation of NMDA receptors in the hippocampus (Caldeira et al., 2007; Levine et al., 1998). BDNF also up-regulates surface expression of

AMPA receptors by inducing their rapid surface translocation to increase excitatory transmission (Narisawa-Saito et al., 2002). Importantly, BDNF has also been reported to facilitate synaptic sensitization of DA neurons in the VTA following cocaine withdrawal. This effect requires the activation of NMDARs and post-synaptic TrkB and, of note, occurs in the presence of $GABA_A$ receptor blocker picrotoxin, suggesting that BDNF can act independently of GABA release to drive activity of VTA DA neurons (L. Pu et al., 2006). It is also plausible that BDNF acts on pre-synaptic glutamate terminals to facilitate excitatory input onto DA neurons by enhancing glutamate release onto DA neurons. In support of this, Numakawa et al. showed that BDNF increases glutamate release in striatal tissue (Numakawa, Takei, Yamagishi, Sakai, & Hatanaka, 1999). Altogether, these data support several possibilities through which BDNF regulates the excitability of VTA DA neurons during food reward related processes. Regardless of how the decreased excitatory activity of the DA neuron is invoked, this model would predict lower average firing rates and decreased accumbens DA transmission, consistent with our $BDNF^{2L/2LCk\text{-}cre}$ mice.

Decreased activation of the NAc may dis-inhibit the LH to promote hedonic feeding

We propose that $BDNF^{2L/2LCk\text{-}cre}$ mice overeat palatable food to compensate for a hypoactive DA circuitry. How might this decrease in activity mediate excessive hedonic feeding? Under burst firing conditions, dopamine secretion results in a net excitatory effect on medium spiny neurons in the NAc (Hjelmstad, 2004). Because these neurons are GABAergic, excitatory afferent activity in the NAc produces feed forward inhibition. This has important consequences maintaining control of food intake, most likely due to connections with the lateral hypothalamus, an important feeding center in the brain.

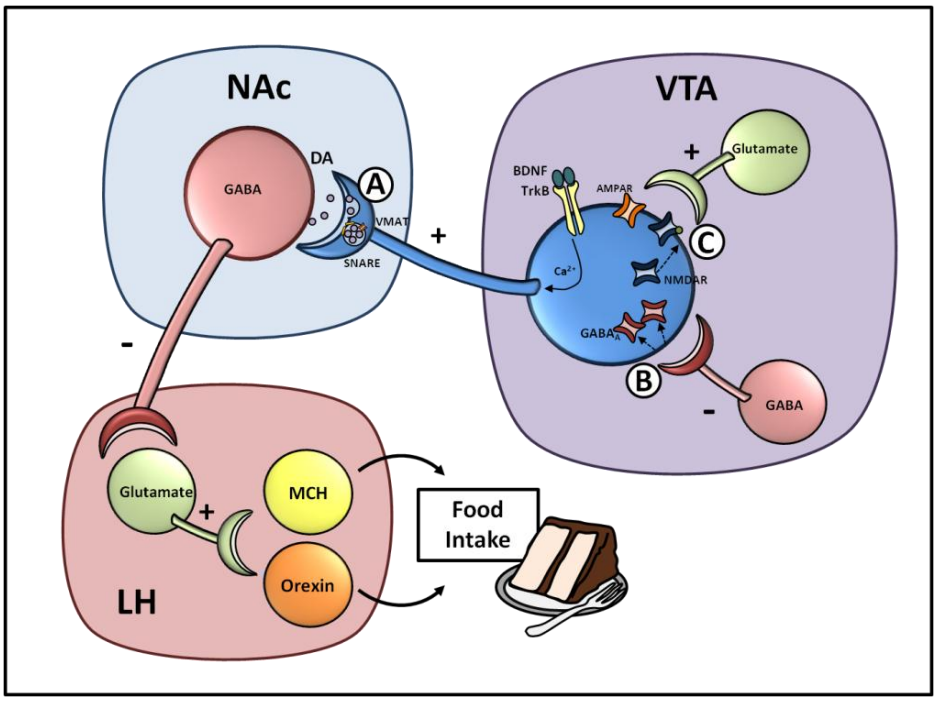


Figure 4.2

A model of BDNF-mediated DA secretion in the NAc and hedonic feeding control via LH connectivity. BDNF signaling in the VTA may facilitate DA exocytosis from VTA DA neurons by **(A)** maintaining synaptic vesicle loading and direct neurotransmitter release mechanisms or by modulating the excitability of these neurons through **(B)** inhibitory or **(C)** excitatory mechanisms. The inhibitory and excitatory drive of VTA DA neurons can be influenced by pre-synaptic neurotransmitter release or modulation of post-synaptic receptors. The result of decreased DA secretion in $BDNF^{2L/2LCk\text{-}cre}$ mice is predicted to be inhibition of NAc GABA neurons which allows LH glutamate neurons to overstimulate orexin/MCH neurons and elicit excessive hedonic feeding.

GABAergic neurons in the shell of the NAc make direct connections to the LH (Heimer, Zahm, Churchill, Kalivas, & Wohltmann, 1991). Injections of the $GABA_A$ agonist, muscimol, in the NAc shell increase feeding and the number of active (c-Fos positive) cells in the LH. Conversely, activation of AMPA receptors in the NAc shell reduces food intake even in food-restricted rats (Stratford et al., 1998). These effects appear to be limited to the action of the NAc shell, since drug application to the core produces little or no effects. Therefore, inhibition of GABAergic neurons in the NAc shell could be responsible for eliciting hyperphagic behavior via the LH.

In support of this, blocking $GABA_A$ receptors in the LH with either picrotoxin or bicuculline (which mimics decreased GABAergic output from the NAc), increases food intake in rats (Kelly, Alheid, Newberg, & Grossman, 1977; Turenius et al., 2009; Turenius, Htut et al., 2009). It is important to note that medium spiny neurons of the NAc do not terminate directly on orexin or MCH neurons in the LH but on glutamatergic neurons projecting to these cells (Sano & Yokoi, 2007). This connectivity is supported by a report demonstrating that delivery of glutamate and AMPA to the LH stimulate intense eating (Stanley, Ha, Spears, & Dee, 1993). Furthermore, the afferent projections most closely associated with the feeding elicited by NMDA in the LH arise from the NAc (Duva et al., 2005). Finally, NMDAR blockade in the LH suppresses NAc-muscimol-induced feeding (Stanley, Urstadt, Charles, & Kee, 2011).

These data collectively support a model in which decreased DA transmission can promote excessive hedonic feeding (Fig 4.2). In short, a decrease in DA transmission in the NAc shell, such as exhibited by $BDNF^{2L/2LCk\text{-}cre}$ mice, would reduce the excitatory drive onto NAc shell medium spiny neurons. This, in turn, is expected to reduce the feed forward

inhibition onto glutamatergic neurons in LH, eliciting hedonic feeding by over stimulating local orexin or MCH neurons (Figure 4.2). This serves as a useful model helping to explain the pathological consumption of palatable food by BDNF mutant mice. It also highlights potential interaction between homeostatic and hedonic control of feeding behavior whereby hunger induced hypothalamic signals may modulate the accumbens reward pathway to increase the drive to eat.

Future Perspectives

How does BDNF influence cell-surface trafficking of α2δ-1?

Alternative splicing of α2δ-1 produces at least 5 known transcriptional variants (Angelotti & Hofmann, 1996). Because BDNF has been reported to promote alternative splicing in the brain (Hayashi et al., 2007) and α2δ-1 contains several domains which contribute to membrane trafficking (Canti et al., 2005), it is possible that alterations in RNA splicing in BDNF mutants might influence these structural features which ultimately impact cell surface expression of α2δ-1.

To begin to identify this possibility, a new high-throughput RNA sequencing technique named RNAseq could be used. In brief, this technique involves reverse transcribing an entire cDNA transcriptome from an mRNA library extracted using its poly(A) tail (which is added to the mRNA molecule post-transcriptionally after splicing has already taken place). The cDNA is subsequently fragmented, sequenced, and aligned to a reference genome for a gene of interest such as α2δ-1 in order to identify variations in RNA content.

Examining the sequence variations of RNA transcripts in the library created from cells in the VMH of WT and BDNF$^{2L/2LCk\text{-}cre}$ mice could not only identify functionally significant splice variations of α2δ-1 transcript in BDNF mutants, it could also discover novel α2δ-1 transcripts in WT mice. In this regard, RNAseq could also help identify whether BDNF mediates membrane targeted α2δ-1 by preferential splicing of known isoforms or presently unidentified transcript variants.

An alternative approach to identify alterations in splicing BDNF$^{2L/2LCk\text{-}cre}$ mice would involve standard PCR and sequencing techniques. Analysis of the entire α2δ-1 cDNA sequence (~7415bp) would require multiple PCR steps. Since several of the natural variations of the transcript are located within or very near the VWA domain, which is critical in the membrane trafficking of α2δ-1, we could begin by exploring potential variations in α2δ-1 transcript produced by BDNF$^{2L/2LCk\text{-}cre}$ mice spanning this region. After RNA extraction and RT-PCR to produce a cDNA library of transcripts expressed by cells in the VMH of WT and BDNF$^{2L/2LCk\text{-}cre}$ mice, we could PCR the sequence spanning the VWA domain with specific primers. Alternative splice variants in this region would generate different sized PCR products, which could be compared between BDNF$^{2L/2LCk\text{-}cre}$ and WT mice by electrophoresis. Specificity of PCR products would be confirmed by DNA sequencing, which would also identify sequence alterations which do not impact transcript length. Like RNAseq, known and novel α2δ-1 splicing variants could be successfully identified by comparing the sequencing results to a reference α2δ-1 cDNA sequence. Together, these studies would help to characterize existing and potentially new α2δ-1 transcripts. They would further identify splice alterations, mediated by BDNF, which are important in the membrane trafficking and function of α2δ-1 protein.

Do α2δ-1:TSP-3 interactions mediate food intake, via synaptogenesis in the hypothalamus?

In Chapter 2, I demonstrated that TSP-3 is regulated by nutritional status in the DMH of WT mice and aberrantly expressed in the DMH of BDNF$^{2L/2LCk\text{-}cre}$ mice who are hyperphagic and obese. It has been reported that TSP-3 deficient mice display transient bodyweight dysfunction, though the mechanism for this is not clear (Hankenson et al., 2010). In light of these data, experiments testing the possibility that TSP-3 mediates food intake would be highly informative. According to our model, TSP-3 from the DMH might interact with α2δ-1 in the VMH to promote excitatory synaptogenesis and increase anorexigenic output.

To begin to examine the effects of TSP-3 on food intake, we could virally deliver TSP-3 to the DMH of wild type mice, the proposed pre-synaptic origin of TSP-3+ fibers interacting with the VMH, and monitor food intake. Our model (Fig 4.1) predicts that increased TSP-3 would increase excitatory synaptogenesis in the VMH, enhancing anorexigenic output to decrease chow consumption. It would also be interesting to examine whether rebound feeding after fasting would be inhibited in mice expressing TSP-3 via AAV-TSP-3. Since viral expression of TSP-3 would not be controlled by the endogenous promoter, levels of virally expressed TSP-3 would be expected to continue to promote satiety during a period of negative energy balance.

Next, in order to test whether TSP-3-mediates effects on food intake via functional interactions with α2δ-1, GP could be administered chronically in AAV-TSP-3 mice. Since GP prevents the interaction between TSP-3 and α2δ-1 and inhibits synaptogenic function

(Eroglu et al., 2009), it would also be expected to prevent TSP-3-mediated decreases in food intake. This could also be explored immunohistochemically by examining the pattern of co-localization for TSP-3 and α2δ-1. Increased TSP-3/α2δ-1 interaction in anorexigenic neurons would be predicted to correspond with decreased food intake.

Lastly, we could examine the synaptic profile of α2δ-1 neurons in the hypothalamus to determine whether TSP-3/α2δ-1-mediated effects on food intake are driven by synaptogenesis and rewiring of synaptic circuits in the DMH and/or VMH. Since the PVN is also a region of high α2δ-1 expression (Cole et al., 2005) and mediates the satiety effects of BDNF in parvocellular neurons (Toriya et al., 2010) which receive excitatory input from the DMH (Bouret, Draper, & Simerly, 2004), it is also possible that α2δ-1 in the PVN is a post-synaptic target for DMH-derived TSP-3 to promote synapse formation and regulate food intake. To examine these possibilities, we could immunolabel and quantify the number and location of excitatory synapses (Vglut2) on α2δ-1 neurons within the VMH, DMH, and PVN of 1) fed versus fasted mice 2) WT versus $BDNF^{2L/2LCk\text{-}cre}$ mice and 3) AAV-TSP-3 versus AAV-GFP treated mice. Increased Vglut2/α2δ-1 immunoreactivity would indicate neurons in a hypothalamic nucleus with increased excitatory drive promoting satiety.

By helping to define the mechanisms underlying the satiety effects of α2δ-1 in the VMH, these studies could identify TSP-3 as a novel factor regulating appetite. Interestingly, since the DMH acts as a putative food-entrainable circadian pacemaker (Gooley et al., 2006; Mieda, Williams, Richardson, Tanaka, & Yanagisawa, 2006), it is also attractive to explore the possibility that TSP-3 expression in the DMH is influenced by time of day to mediate synaptic connectivity between the VMH and/or PVN to control circadian feeding patterns. Indeed, it has been previously suggested that the synaptic organization of the melanocortin

system undergoes circadian oscillation in line with circadian feeding and metabolic patterns (Horvath, 2005). Consistent with this hypothesis, serum TSP-1 levels oscillate in a circadian manner in humans with the lowest levels occurring in the morning (effectively after an overnight of fasting) (Tan, Syed, Lewandowski, O'Hare, & Randeva, 2008). In mice, I have observed that while WT mice consume 80% of their total food intake during the nocturnal period, $BDNF^{2L/2LCk\text{-}cre}$ mice with altered expression of TSP-3 in the DMH consume only 60% (n=4, $p = 0.03$; unpublished data). This suggests a potential dysregulation of circadian feeding in $BDNF^{2L/2LCk\text{-}cre}$ mice, which may be linked to the increased TSP-3 transcript levels they exhibit in the DMH. To examine this possibility more closely, it would be interesting to measure the levels of TSP-3 in the DMH throughout the circadian cycle by Western blot and examine the corresponding synaptic profiles in DMH targets such as the VMH and PVN in WT and $BDNF^{2L/2LCk\text{-}cre}$ mice. Together, data from these studies could help to validate a synaptic model for regulating food intake in the hypothalamus and also help define mechanisms through which the DMH mediates circadian control of feeding.

Does α2δ-1 exist independently from α1 at the cell surface of VMH neurons?

VMH neurons in BDNF mutant mice do not show alterations in whole-cell Ca^{2+} current, decay kinetics, or voltage gating. Since these measures of α1 function are mediated by α2δ-1 (A. Davies et al., 2007), our data suggest that the amount of α2δ-1 in complex with α1 at the surface of VMH neurons is similar for both $BDNF^{2L/2LCk\text{-}cre}$ and WT mice. Importantly, however, these neurons in $BDNF^{2L/2LCk\text{-}cre}$ exhibit a significant decrease in cell surface expression of α2δ-1. Since α2δ-1 is not always found in association with α1 (Garcia

et al., 2008), these data are consistent with the possibility that a surface population of α2δ-1 existing independently from α1 is significantly reduced in the $BDNF^{2L/2LCk\text{-}cre}$.

To begin to test the possibility, we could immunolabel α2δ-1 and α1 in neurons of the VMH of wild type and $BDNF^{2L/2LCk\text{-}cre}$ mice and assess the level of co-localization. It would also be possible to use co-immunoprecipitation to study the extent of α2δ-1 and α1 in physical association at the cell surface. For this, biotinylated surface protein from these cells could be immunoprecipitated with avidin beads. Using this surface protein fraction, a second immunoprecipitation onto α1 beads would discriminate α1-bound and extra-α1 protein extracts, which could subsequently be probed with anti-α2δ-1 by Western blot. Whereas WT and $BDNF^{2L/2LCk\text{-}cre}$ mice would be expected to have similar amounts of α2δ-1 and α1 in complex, it would be predicted that VMH neurons in $BDNF^{2L/2LCk\text{-}cre}$ mice would have less α2δ-1 independent of α1.

In response to a decrease of surface α2δ-1, it is alternatively possible that a compensatory upregulation of α2δ-2 and α2δ-3 contributes to the trafficking and maintenance of Ca_V function. However, under basal conditions the expression of α2δ-2 and α2δ-3 are extremely low in the VMH (Cole et al., 2005). Since mice with mutations of α2δ-2 with decreased surface expression do not show compensation by other α2δ subunits (Barclay et al., 2001), the possibility for compensation seems minimal. However, to directly test this possibility, RNA extracted from VMH cells in WT and $BDNF^{2L/2LCk\text{-}cre}$ mice could be analyzed using quantitative RT-PCR with α2δ-2 and α2δ-3 specific primers. Surface levels of α2δ-2 and α2δ-3 protein could be assayed with biotinylation and Western blot. Together, these studies would help to expand our understanding of the relationship between α2δ-1 and α1 as well as identify a potential role for α2δ-1 at the cell surface independent of α1.

How does BDNF mediate evoked DA secretion in the NAc?

Amperometry studies revealed decreased evoked secretion of DA in the NAc in $BDNF^{2L/2LCk\text{-}cre}$ mice. Several possible mechanisms exist through which BDNF signaling might regulate DA activity and the secretion of DA. First, BDNF might regulate neutrotransmitter release mechanisms. This is supported by studies identifying deficits in SNARE proteins and VMAT activity in BDNF knockout and heterozygous mice (Boger et al., 2011; Jovanovic et al., 2000; Pozzo-Miller et al., 1999). To test whether BDNF might regulate DA activity by directly controlling DA release mechanisms, we could measure transcript levels of synaptobrevin, synaptophysin, and synapsin in the VTA of WT and $BDNF^{2L/2LCk\text{-}cre}$ mice using quantitative RT-PCR. Since these proteins are located at synaptic terminals, a Western blot quantifying protein levels should be performed using lysate extracted from the NAc. Decreased expression of these transcripts or proteins could contribute to the deficits in evoked DA secretion observed in the NAc of $BDNF^{2L/2LCk\text{-}cre}$ mice.

Excitatory and inhibitory inputs converging on DA neurons also mediate burst firing and the release of DA from the VTA. Thus, deficits in evoked secretion of DA in the NAc of $BDNF^{2L/2LCk\text{-}cre}$ mice may also result from dysregulation leading to decreased excitation or increased inhibition of DA neurons in the VTA. One way to examine whether BDNF mediates the excitatory activity of DA neurons is to measure spontaneous, mini excitatory post-synaptic currents (EPSCs) in VTA DA neurons from WT and $BDNF^{2L/2LCk\text{-}cre}$ mice. In the presence of TTX, which blocks synaptic events caused by action potentials, a decrease in the amplitude of mini EPSCs in $BDNF^{2L/2LCk\text{-}cre}$ mice would indicate a post-synaptic defect within VTA DA neurons, leading to decreased excitation and decreased DA release.

However, a decrease in the frequency of mini EPSCs could indicate either a deficit in pre-synaptic release of glutamate onto DA neurons or a decreased post-synaptic response in DA neurons. To examine the post-synaptic mechanisms more closely, and because BDNF has established roles in the translation, trafficking, and phosphorylation of NMDA and AMPA receptors (Caldeira et al., 2007; Levine et al., 1998; Narisawa-Saito et al., 2002), I would examine whether total and phosphorylated levels of these receptors in VTA protein lysates are decreased in $BDNF^{2L/2LCk\text{-}cre}$ mice. This could be accomplished by Western blot. Alternatively, surface expression of these receptors could be analyzed using a biotinylation assay. Decreased levels of these receptors in $BDNF^{2L/2LCk\text{-}cre}$ mice would be consistent with a post-synaptic deficit in VTA DA neurons contributing to decreased excitation. Pre-synaptic deficits, such as impaired glutamate release onto VTA DA neurons, could be more closely examined by measuring the paired-pulse ration (PPR) in whole-cell evoked EPSCs. A decreased PPR in DA neurons from the VTA of $BDNF^{2L/2LCk\text{-}cre}$ mice compared to WT would indicate a pre-synaptic deficit in glutamate release onto DA neurons, consistent with the possibility that decreased excitation is reducing DA secretion.

It is alternatively possible that increased inhibition of VTA DA neurons contributes to decreased evoked DA secretion in the NAc of $BDNF^{2L/2LCk\text{-}cre}$ mice. To investigate this possibility, we could measure spontaneous, mini inhibitory post-synaptic currents (IPSCs). An increase in only the amplitude of mini IPSCs measured in DA neurons in VTA slice preparations from $BDNF^{2L/2LCk\text{-}cre}$ mice would indicate a post-synaptic mechanism mediating DA neuron excitability. Since BDNF is known to promote internalization of the $GABA_A$ from the cell membrane (Hewitt & Bains, 2006), a biotinylation assay examining $GABA_A$ in DA neurons in the VTA of $BDNF^{2L/2LCk\text{-}cre}$ mice might reveal a surplus of receptors at the

cell-surface. This would identify a potential post-synaptic alteration in DA neurons which would decrease excitability. However, a pre-synaptic mechanism increasing GABA-mediated inhibition of VTA DA neurons could also decrease DA release. This possibility would be supported by an increase in the frequency of mini IPSCs and more directly by an increased PPR measured in VTA DA neurons. Altogether, these experiments would significantly improve our knowledge of the mechanisms through which BDNF mediates DA activity in the mesolimbic pathway and the alterations in BDNF mutant mice which contribute to decreased excitability of VTA DA neurons and excessive consumption of palatable food.

Does decreased activation of the NAc promote hedonic feeding via functional connectivity with the LH?

Dopamine secretion results in a net excitatory effect on GABAergic neurons in the NAc shell (Hjelmstad, 2004) which make connections to the LH (Heimer et al., 1991), a well established feeding center in the brain. LH glutamate neurons receiving inhibitory input from the NAc are suggested to mediate local activity of orexin and MCH neurons (Sano & Yokoi, 2007). Thus, decreased DA tone in the NAc of BDNF mutant mice may promote hedonic feeding by decreasing feed-forward inhibition onto the LH. To begin to test this model (Fig 4.2), we could immunolabel for alterations in c-Fos in the LH of WT and BDNF mutant mice. Since c-Fos is upregulated during recent neuronal activity, it would serve to identify activated neurons in the LH. Double immunolabeling for c-Fos/orexin or c-Fos/MCH in WT and $BDNF^{2L/2LCk\text{-}cre}$ would be even more informative, since activation of

these neurons has an established role in promoting food intake. Inactivation of the NAc shell enhances orexin neurons in the lateral hypothalamus (Baldo et al., 2005). Thus, it is predicted that $BDNF^{2L/2LCk\text{-}cre}$ mice will have increased c-Fos/orexin and c-Fos/MCH immunoreactivity.

A pharmacological approach testing our model would have the advantage of examining the connection between the NAc and the LH at the behavioral level. For this, I would cannulate and monitor food intake in $BDNF^{2L/2LCk\text{-}cre}$ mice after selectively administering drugs to influence the activity of 1) GABA neurons in the NAc, 2) glutamate neurons in the LH, and 3) Orexin/MCH neurons in the LH. To first determine if excessive feeding in $BDNF^{2L/2LCk\text{-}cre}$ mice is the result of inhibited GABA neurons in the NAc, I would site-specifically deliver D1 agonists to the NAc shell. It is predicted that this would excite medium spiny neurons, allowing proper feed-forward inhibition of glutamate and orexigenic neurons in the LH. Thus, $BDNF^{2L/2LCk\text{-}cre}$ mice with delivery of D1 agonists to the NAc shell would presumably decrease palatable food intake. This is consistent with reports demonstrating that delivery of GABA agonists to the NAc shell of WT mice promotes feeding, by inhibiting the inhibitory output of GABA neurons (Stratford et al., 1998).

To examine whether the increased glutamatergic activity in the LH contributes to the hyperphagic behavior of $BDNF^{2L/2LCk\text{-}cre}$ mice, I would cannulate and deliver the GABA agonist, muscimol, to the LH. By mimicking normal GABAergic innervations of the LH, muscimol ought to inhibit the activity of glutamate neurons and reduce the orexigenic output of the LH, decreasing food intake in $BDNF^{2L/2LCk\text{-}cre}$ mice. This outcome is supported by reports that $GABA_A$ blockade in the LH increases food intake in rats (Kelly et al., 1977; Turenius et al., 2009; Turenius, Htut et al., 2009).

Finally, to test the possibility that increased excitation of orexigenic neurons in the LH increases palatable food intake in $BDNF^{2L/2LCk\text{-}cre}$ mice, I would deliver glutamate receptor antagonists to the LH of these mice. Similar to studies demonstrating that delivery of NMDA receptor antagonists in the LH suppresses NAc-muscimol-induced feeding (Stanley et al., 2011), NMDA or AMPA antagonism in the LH of $BDNF^{2L/2LCk\text{-}cre}$ mice would be predicted to reduce food intake by inhibiting the excitation of orexin and MCH neurons. This is also consistent with reports that delivery of NMDA and AMPA agonists to the LH stimulates feeding in rats (Stanley et al., 1993).

In short, literature directly supports a model whereby decreased DA transmission in the NAc shell reduces feed-forward inhibition onto glutamatergic neurons in LH, eliciting hedonic feeding by stimulating local orexin or MCH neurons. Testing this model using these immunohistochemical and pharmacological approaches would offer valuable insight into the mechanisms producing hedonic eating in $BDNF^{2L/2LCk\text{-}cre}$ mice.

Summary

In conclusion, the work described in this thesis significantly enhances our understanding of the mechanisms mediating the anorexigenic actions of BDNF in the brain. In the VMH, I established a novel role for the calcium channel subunit and thrombospondin receptor, α2δ-1, facilitating BDNF in the regulation of food intake and energy homeostasis. I also identified a novel capacity for BDNF to control hedonic feeding of high-fat, palatable food by positively modulating the mesolimbic dopamine system. The results of these studies reveal exciting, new therapeutic targets with direct implications for clinical health. This is

especially true given the high percentage of individuals harboring mutations which interfere with BDNF signaling and the increasing global prevalence of obesity. As we endeavor to develop novel and safe treatment strategies for obesity and its associated medical complications, it is critical that we continue to explore and define the mechanistic details of BDNF signaling in these key neural circuits mediating food intake control.

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