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Trends Endocrinol Metab. Author manuscript; available in PMC 2021 January 01.

Published in final edited form as:

Trends Endocrinol Metab. 2020 January ; 31(1): 46–52. doi:10.1016/j.tem.2019.09.001.

Neuronal Cell Cycle Events Link Caloric Intake to Obesity

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Abstract

Obesity is a neurological disorder which operates by favoring energy storage within adipose depots and increased caloric intake. Most cases of human obesity are acquired without any underlying genetic basis. We suggest that obesity can impair the function of some hypothalamic neurons critical to body weight regulation. Genetic ablation of the retinoblastoma gene within pro-opiomelanocortin neurons leads to death of the neurons and subsequent obesity. The retinoblastoma protein (pRb), a key inhibitor of the cell cycle, can also be inactivated by CDK-mediated phosphorylation. Extensive development led to the production of FDA-approved CDK4/6 inhibitors. Based on our own results, we propose that maintaining or re-instating pRb function using CDK4/6 inhibitors are potentially effective treatments of diet-induced obesity

Keywords

Cell cycle; Diet-induced Obesity; Hypothalamus

Diet-induced obesity: an incomplete picture

Obesity is one of the most prevalent threats to human health: the current adult obesity rate in developed countries is 19.5%, with a staggering 38.2% obesity rate in the United States alone [1]. New insights into the etiology of this pandemic are desperately required in order to introduce successful therapeutic options in the near future. In the twenty-five years since the discovery of the “satiety hormone” leptin [2, 3], work in the field has progressively identified key cellular and molecular determinants of energy balance regulation. However, early studies in obese humans [4] found no evidence to support any obvious genetic defects in the leptin pathway, a finding which still holds true in recent high throughput genetic

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screens [5, 6] With the molecular etiology of obesity remaining unclear in the context of these genetic studies, it has been hypothesized that the rising prevalence of obesity in the developed world can be largely attributed to environmental factors such as decreased energy expenditure and increased availability of calorie-dense foods. The most widely used laboratory rodent model of leptin-replete obesity utilized the administration of a high-fat diet (HFD, in which 60% kilocalorie content was derived from fat), and was subsequently characterized as diet-induced obesity (DIO) [7]. However, early work to determine the etiology of DIO reached contradictory conclusions. Several studies reported a phenomenon of HFD-induced hyperphagia, in which HFD was shown to either increase overall caloric intake, directly contributing to obesity due to an increase in positive energy balance [8, 9]. However, a near equivalent number of studies reported a complete absence of this hyperphagic phenotype during HFD feeding [10, 11]. Molecular genetic studies have reached similarly contradictory conclusions—early studies of HFD effects on the hypothalamus showed a modest HFD-induced decrease of LEPR-B (B isoform of leptin receptor) expression in the mediobasal hypothalamus (MBH) of C57BL/6J mice, proposing a "decreased available receptor quantity" model of leptin resistance [12]. However, more recent studies of the effects of DIO on differential gene expression in global hypothalamic neurons [13] and specifically anorexigenic POMC neurons [14] reached the opposite conclusion, demonstrating either no gene expression changes [13], or a modest HFD-mediated *increase* in both *Lepr-b* and *Socs3* expression [14]—both suggesting the HFD-mediated "leptin resistance" in MBH neurons cannot be attributed to lower leptin receptor expression level. One of the most striking conclusions of these studies was that "leptin sensitivity" could be functionally restored in DIO mice by simply switching from HFD to standard chow diet, with returning levels of leptin sensitivity directly correlated to loss of fat mass [13].

Pathophysiology of HFD-induced damage to energy balance neurons

The mediobasal hypothalamus is a key central regulator of energy balance and comprises the arcuate nucleus (ARC) and the ventromedial nucleus (VMN). The ARC contains two antagonistic neuron populations, the anorexigenic pro-opiomelanocortin (POMC) and the orexigenic agouti-related peptide/Neuropeptide Y (AgRP/NPY) neurons [15]. Elevated leptin levels during a positive energy state simultaneously activates POMC neurons and inhibits AGRP neurons though the leptin receptor (LEPR). A physiologically appropriate response in these two cell types to leptin signaling is critical for maintaining energy balance [16, 17], and thus, there has been much speculation around their sensitivity to changes in dietary intake. Moreover, ARC neurons are anatomically adjacent to the Median Eminence (ME), the inferior boundary of the hypothalamus and site of hormone release into the hypophyseal portal system [18], making it one of areas of the brain devoid of a blood-brain barrier with ME-adjacent neurons being accesssible to circulating factors. Indeed, it has been shown that parenteral administration of agents such as monosodium glutamate can result in the selective destruction of ARC neurons due to direct exposure [19]. Additional relevant studies have shown that HFD leads to increased serum free fatty acids (FFA), which can be directly sensed by hypothalamic neurons [20]. Integrating these studies suggests that ARC-ME neurons may be selectively vulnerable to changes in serum factors in response to

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HFD intake. Indeed, it has been shown that HFD intake leads to an acute rise in both inflammatory mediators and gliosis markers in the rodent ARC in response to only several days exposure to HFD—well before the onset of obesity; the same study demonstrated an increased level of gliosis in the MBH of live obese humans [21]. Further studies in the role of neural inflammation in DIO demonstrate that activation of hypothalamic inflammatory pathways such as inhibitor of nuclear factor-κβ kinase-(IKKβ) or nuclear factor-κβ (NF-κβ) promotes a rapid hyperphagic response and weight gain, and effectively desensitizes the leptin responsiveness of MBH neurons [22]. Additional studies confirmed this effect was mediated by high fat diet intake (specifically elevated fat macronutrient composition and *not* caloric content) and reversing the hypothalamic inflammation subsequently restored central leptin and insulin responses—again indicating the existence of a direct and *reversible* HFD-neural injury pathway [23]. An alternate line of studies has shown hypothalamic neurons experience an endoplasmic reticulum (ER) mediated stress response during obesity, and that the reversal of ER stress can restore leptin sensitivity in DIO mice [24]. The implications of the ER-stress model are in part concordant with the inflammatory/gliosis model, in that both demonstrate leptin-independent pathways of MBH neuron damage brought on by environmental models of obesity.

Cell Cycle Events as a Biomarker of Neuronal Damage

Postulating that DIO can be the result of direct MBH neuronal injury underlies two key unknowns to the translational significance of this hypothesis: what the molecular basis of neuronal injury is, and how it can be targeted for reversal in obese patients. Recent work proposes cell cycle regulation (Box 1) as both a biomarker and druggable target for the neuronal damage causal to obesity, demonstrating unexpected sites of *de novo* neurogenesis, aberrant neuronal cell-cycle reentry and DNA replication, and dysregulation in neurons in response to a variety of toxic environmental stimuli.

While entry from interphase into mitosis is required for the propagation of stem/progenitor cells such as hypothalamic neural stem cells (htNSCs), most fully-differentiated cells are not destined for any further self-replication and are termed “post-mitotic”. Adult neurons are the archetypical example of this post-mitotic cell fate, which is an interphase state of cell cycle withdrawal known as G_0 [25]. As such, a widely held belief is that adult neurons do not have the ability to reenter cell cycle, and thus cannot self-replicate. However, a substantial body of recent work has demonstrated nuances in the permanence of the neuronal G_0 state, particularly during states of cellular stress or injury [26]. A prevailing hypothesis in this area is that neuronal quiescence is not a terminal fate *per se*, but rather a state of constant vigilance that is actively regulated by cyclin kinase inhibitors (CKIs) and other intracellular factors; “relaxation” of this vigilance leads to cell-cycle reentry, which can manifest as neuronal dysfunction along a continuum of severity, and can eventually result in cell death if not reversed [27]. Factors which have been shown to induce neuronal cell cycle reentry include DNA damage, which leads to induction of CDK4/cyclin D and eventual neuronal death, but can be rescued with constitutively active CKIs p16, p21, and p27 [28]. Oxidative stress has been additionally implicated as an environmental factor which can dysregulate neuronal cell cycle, and in fact has been repeatedly shown to be a pathology which precedes neuronal apoptosis in several human neurodegenerative diseases [29]. These cell cycle

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reentry events are usually catastrophic and irreversible, and lead to activation of an E2F1 mediated apoptosis pathway directly after G_1 entry, termed “abortive cell cycle reentry” [30, 31].

Interestingly, emerging research indicates that not all neuronal cell-cycle reentry events are unproductive and fated for apoptosis: evidence shows neurons can successfully undergo S phase and exist in a tetraploid state of stable pre-mitotic G_2, although it is unclear what degree pre-replication neuronal function remains. On one end of the spectrum, tetraploidy in a terminally-differentiated neuron forecasts inescapable cell death; for example, hypoxic-ischemic stress leads to cell-cycle reentry and completion of S phase in rodent hippocampal neurons, but these neurons rapidly undergo apoptosis soon after [32]. On the other end of the spectrum, there is evidence of stable somatic tetraploidy in fully functional adult neurons, such as retinal ganglion cells induced to re-enter cell cycle using nerve growth factor. In this scenario tetraploidy creates no measurable detriment on cell function, and these cells exist in their functional tetraploid state throughout adulthood [33]. For the most part, the majority of identified tetraploid adult neurons seem to have some degree of either functional deficit or increased vulnerability.

Using cell cycle reentry as a disease hallmark in neurodegenerative disease not only underscores the importance of cell cycle vigilance in neuron function, but also uncovers possible therapeutic options towards restoring neuron function. Similar to AD patients, a certain degree of cell cycle reentry is seen in the dysfunctional neurons of PD patients as well, as evidenced by DNA content, pRb phospho-inactivation, and E2F1 target gene expression [34]. Remarkably, small-molecule inhibition of CDK4 by the pan-CDK inhibitor flavopiridol has been shown to have neuroprotective effects in PD models, and even attenuate apoptosis in re-entrant neurons [35]. This provides compelling evidence that while cell cycle reentry is catastrophic for neuron function, this process can be targeted by drug therapy and may be reversible.

As cell cycle reentry has been shown to be a cause of neuron functional failure, in the context of DIO, the question again arises if elevated dietary fat intake can be a trigger for this damaged neuronal state. As discussed earlier, elevated dietary fat intake can certainly trigger neuronal stress states through inflammatory and other mitogenic pathways. Thus, whether elevated dietary fat intake can generate enough neuronal stress to result in a cell cycle reentry event is by no means a far-reaching speculation. However, there is scant evidence that high fat feeding leads to active proliferation of neurons. Rather, a more molecularly focused aspect of our hypothesis is the induction of E2F target gene expression that could generate aberrant neuronal functions. It is likely that there may be a small suite of genes, which remain to be identified, that fit this description.

Studies which have attempted to look at the role of HFD on the MBH have shown a direct suppression of htNSC neurogenesis in response to HFD, which was phenocopied in *ob/ob* mice, demonstrating a htNSC replication deficiency may in part be causal to obesity [36]. A similar study which further integrates the HFD-mediated neuron inflammatory response with HFD effects on htNSCs replicated the finding that chronic HFD administration leads to both the depletion and neurogenic impairment of htNSCs, but went on to directly link this

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phenomenon to selective IKKβ/NF-κB activation mediated apoptosis in the htNSCs population [37]. However, not all studies in this area have had concordant results: another line of work looking into the role of htNSCs in the ARC-ME found that while neurogenesis was in fact sensitive to dietary intake, HFD feeding actually increased neurogenesis in this area; furthermore, selectively inhibiting neurogenesis in the ventrobasal hypothalamus prevents HFD-induced weight gain [38]. This may suggest that elevated MBH neurogenesis alters the ratio of positive to negative energy balance neurons in an obesity-promoting manner. In short, it remains unclear whether HFD induces activation or prevention of neurogenesis, as does the role of hypothalamic neurogenesis in DIO.

Together, these studies suggest HFD-mediated cell cycle injury in the MBH may be a "two-prong" effect: (A) adult post-mitotic MBH neurons experience HFD-mediated stress, initiating a transcriptional response triggered by activated E2Fs, compromising neuron function, and possibly slowly undergoing apoptosis, while the (B) htNSC population, which would otherwise replace this depleted pool of adult MBH neurons, experiences impaired neuronal differentiation due to HFD. Although it is unclear whether HFD-related neurogenesis protects against or accelerates DIO, it is clear that altered dietary intake can certainly perturb the baseline htNSC replication dynamic. Combined, these events alter the functional population of MBH neurons leading to a deficiency in melanocortinergic transmission and DIO.

The retinoblastoma protein pathway as a druggable target for DIO

The effect of HFD on adult ARC-ME neuron cell cycle remains unclear. One of the earliest reports of the effects of HFD on adult ARC-MRE demonstrated that chronic HFD feeding leads to phosphorylation of pRb (Box 2) in the MBH (including in POMC neurons), and that these neurons re-entered cell cycle as measured by E2F1 target gene expression; pRb's role as an "obesity suppressor" made it critical in POMC neurons but dispensable in AgRP neurons [39]. Interestingly, pRb is not the only cell cycle regulator affected by HFD in the MBH. A very recent study demonstrated that the tumor suppressor p53 also plays a major role in the prevention of DIO—the loss of p53 function specifically in AgRP neurons increases vulnerability to DIO by increasing food intake during HFD feeding, suggesting that p53 is required for feeding adaptations against DIO [40]. It is interesting to note that while these antagonistic neuron populations can both demonstrate compromised function in response to HFD-induced cell cycle stress, they have a differential preference between p53 and pRb as their respective master regulators.

A report [41] directly tested the possibility of reversing/preventing diet induced obesity from HFD using two methods: A) expression of a non-phosphorylable pRb in which 18 potential phosphorylable Ser/Thr residues had been altered to alanine and B) treatment with a CDK4/6 inhibitor (Box 3) to prevent pRb inactivation. Virally delivered expression of the nonphosphorylable pRb, within the mediobasal hypothalamus or within ARC POMC neurons only, prevented excess fat mass gain in C57BL/6J mice on HFD. Abemaciclib, a CDK4/6 inhibitor, was effective in both preventing and reversing HFD-induced obesity with a significant reduction of fat content without affecting fat-free mass. Significantly, control animals on the same high fat diet that underwent a similar degree of weight reduction by

food restriction exhibited loss of lean mass and fat mass, suggesting that inhibition of CDK4/6 promoted lipolysis as the principal mode for weight loss. Another significant finding is that DIO is reversible with abemaciclib treatment, suggesting that the energy homeostatic system is not permanently damaged and could be rendered functional with appropriate treatment. A final finding was that mice on normal chow were not affected by abemaciclib, supporting the notion that weight loss due to CDK4/6 inhibition was due to normalization of the function of the weight-regulatory system rather than being a pharmacological effect of drug treatment. These findings are in contrast to the mouse model wherein *Rb* deletion in POMC neurons led to irreversible loss of POMC neurons. The differences between the models could be due to influences of *Rb* deletion during neuronal development or incomplete inactivation of pRb by CDK4/6. Further studies with long term feeding of the HFD will be needed to determine if POMC neurons are lost in significant numbers and whether pRb is involved in the process.

Concluding Remarks

We have provided a framework for approaching obesity as an environmentally induced disorder of cell cycle control in hypothalamic neurons. The identification of phospho-inactivated retinoblastoma protein in these neurons implicates cyclin dependent kinases as instigators of the process leading to neuronal dysfunction. As pRb is turned over, inhibition of CDKs leads to the reinstatement of pRb function and potential reversal of hyperphagia and obesity. The further exploration of fundamental cellular mechanisms in regulating neuron health will provide insights to novel therapeutic modalities for obesity and other metabolic disorders.

Acknowledgements

Funding: This work was funded by NIH grant 5 R01 DK111043 to SC and LZ, and F30 DK116532 to NJI. Imaging analysis was supported by the Einstein Analytical Imaging Facility, NCI Cancer Center Support grant P30CA013330. Additional support and resources were provided by Einstein Sinai Diabetes Research Center Grant P60DK020541, the New York Obesity Research Center Grant 1P01DK026687, and Liver Pathobiology and Gene Therapy Research Core Center Grant 5P30-DK041296.

Glossary

ARC arcuate nucleus of the hypothalamus

AgRP agouti gene related peptide

CDK cyclin dependent kinase

CKI cyclin dependent kinase inhibitor

DIO diet induced obesity

htNSC nypothalamic neural stem cells

HFD high fat diet

LEPR leptin receptor

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MBH	medio-basal hypothalamus
ME	median eminence
NFkappaB	nuclear factor kappa light chain enhancer of B cells
NPY	Neuropeptide Y
POMC	pro-opiomelanocortin
Rb	retinoblastoma
VMH	ventromedial hypothalamus

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An overview of the cell cycle

The eukaryotic cell cycle is divided into two major phases: the mitotic state of cell division, and interphase. Interphase is further subdivided in three stages: G_1, when the proteins responsible for DNA replication are synthesized, S phase, when nuclear DNA is replicated, and G_2, when the proteins responsible for cell division are synthesized. Progression through interphase is tightly regulated by a class of proteins known as cyclins and partner cyclin-dependent kinases (CDKs): Cyclin D is synthesized at the beginning of G1, where it activates CDK4/6 to phosphorylate retinoblastoma protein (pRb) and activate the transcription factor E2F1, which induces the synthesis of the proteins necessary for DNA replication[42]. Full progression to S phase and DNA replication is completed via the successive associations of cyclin E and cyclin A to CDK2, which phosphorylate additional residues of pRb[43]. A cyclin A/CDK1 complex activates late replication origins during G_2 phase, inducing thecondensation of chromosomes prior to mitotic entry [44]. An additional class of regulatory proteins counteracts cyclin/complexes to negatively regulate cell cycle and are accordingly called Cyclin Kinase Inhibitors (CKIs); there are two major classes of CKIs, namely the Ink and Cip/Kip families. The Ink family proteins $p15_{Ink4b}$, $p16_{Ink4a}$, $p18_{Ink4c}$, and $p19_{Ink4d}$ are the major regulators of entry to G_1 state: they actively bind CDK4/6 and block cyclin D association, preventing pRb phospho-inactivation and E2F target gene expression [45]. The Cip/Kip family of $p21_{Cip1}$, $p27_{Kip1}$, and $p57_{Kip2}$proteins function on a more global scale throughout interphase, and are able to inhibit multiple CDK/cyclin complexes during interphase [46]. Interestingly, a population of this progenitor population is found in the MBH, denoted hypothalamic neural stem cells (htNSCs), suggesting that there may be *de novo* neurogenesis in the MBH in response to environmental stimuli or in order to accommodate neuronal turnover [47].

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The retinoblastoma protein

Whether cell cycle progression is occurring in adult or progenitor neuron populations, the master regulator of this process is both cell types is the retinoblastoma protein (pRb). The core function of pRb is to bind and repress the E2F family of transcription factors, preventing cell cycle progression into S phase [48]. The bi-allelic loss of pRb inappropriately accelerates cell proliferation, and as expected, leads to tumorigenesis in several tissue types, including the eponymous retinoblastoma [49]. pRb is additionally often cited as the classical examples of a "two-hit tumorigenesis" model: while pRb is haplosufficent to prevent tumorigenesis in heterozygous individuals, a second genetic insult to the functional copy of RB1 (or loss of heterozygosity), statistically pre-disposes heterozygotes to developing early retinoblastoma, as well as other cancers in later life [50]. Finally, pRb function can be blocked at the protein level to drive tumorigenesis. Several oncovirus gene products directly target pRb, most often through direct binding and degradation of pRb; one of the most well studied clinical examples of this phenomenon is the human papillomavirus (HPV) protein E7 [50]. The second method by which "genetically intact" pRb can be functionally inactivated is through phosphorylation: pRb is an extensively phospho-regulated protein, as discussed below.

There are 15 known consensus phospho-acceptor sites for CDK-Cyclin mediated phosphorylation (S/TPxK/R motif) in pRb [51-53]. A recent study used 2-D isoelectric focusing electrophoresis to reveal the relationship between extent of pRb phosphorylation and its functional inactivation. When quiescent fibroblasts were stimulated to re-enter cell cycle to early and mid G_1 pRb became mono-phosphorylated on any one of the 15 consensus S/Ts by cyclin D/CDK4. Mono-phosphorylated pRb remained bound to E2F but allows cyclin E/CDK2 to phosphorylate a total of 12 or more of the 15 consensus S/Ts. This degree of *hyper*-phosphorylation inactivates pRb to activate E2F for target gene expression and completes entry into S phase [54]. Thus CDK4-cyclin D can initiate pRb phosphorylation, but functional inactivation requires *hyper*-phosphorylation of at least 12 CDK consensus S/Ts. Consequently, this hyper-phosphorylation cascade can be prevented by CDK4 blockade, establishing a "druggable" target for aberrant pRb-inactivated cell-cycle reentry.

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Cyclin-dependent kinase inhibitors

In tumorigenesis, pRb can be genetically unaltered but functionally inactivated by hyper-phosphorylation by CDKs. This aberrant CDK activity is usually secondary to a genetic loss of a CKI such as p16 or p21 in cancer cells [55].pRb phosphorylation is initiated by cyclin D/Cdk4 and completed by cyclin E or A/CDK2 or 1 [51-53]. As such, a modern cancer therapy rationale has been to directly block the activity of CDKs, to "re-activate" pRb in tumor cells and prevent tumorigenic cell-cycle activity [56]. While early pan-CDK inhibitors showed serious side effects, CDK4/6 selective inhibitors have shown remarkable efficacy in treating cancers with WT *RB1* genetic background [56, 57], indicating pRb can be functionally reactivated by CDK4/6 inhibition. The FDA approved CDK4/6 inhibitor Palbociclib for treatment of $ER^{+}/HER2^{-}RB1^{wt}$ breast cancer in 2015, and new inhibitors Ribociclib and Abemaciclib in 2017 [57, 58].

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Outstanding Questions

1. Is there a unique neuronal signature of E2F1 target genes that are induced by high fat feeding?

As pRb mainly acts via inhibition of E2F1, the disinhibition of E2F1 by pRb inactivation would lead to a set of genes whose expression would be induced. A set of E2F1 regulated genes that is unique to neurons could provide a potential set of drug targets with CNS specificity.

2. What are the mechanisms by which pRb inactivation leads to neuronal dysfunction?

Specifically, POMC neurons are dysfunctional after pRb inactivation. However, the molecular and specific cellular deficits caused by an inactive pRb remain unknown. Specific tests for sensitivity to hormones/nutrients could address the ability of POMC neurons to sense environmental cues as well as specific signal transduction pathways while testing of neurons downstream of POMC neurons could address connectivity issues.

3. Is the reversibility of high fat diet induced obesity by CDK4/6 inhibition constrained within a critical period?

It is possible that the mechanism for causing dysfunction in POMC neurons can cause irreversible cellular damage after a certain period of time. However, even in the most severe case of genetic obesity, complete leptin deficiency, leptin is able to reverse the obese phenotype, suggesting that irreversible damage to the brain's weight-regulating system has not occurred even after lifelong obesity.

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Highlights:

Genetic studies have identified numerous genes and neural circuits that cause hyperphagia and obesity although the majority of obese humans have no identifiable mutations in these genes. Diet-induced obesity (DIO) with a calorically dense diet indicates environmental factors directly perturb the functional status of the energy balance neuronal circuitry.

Adult neurons, terminally differentiated and mitotically quiescent, can be triggered to re-enter the cell cycle due to elevated cellular stress. This process leads to impaired neuronal function and neurodegeneration.

Studies approaching obesity as a neuronal cell cycle disorder have revealed the critical role of the retinoblastoma protein (pRb) in maintaining neuronal health. Novel therapeutic options are available with repurposing of drugs developed to treat cancers dependent on inactivation of pRb.

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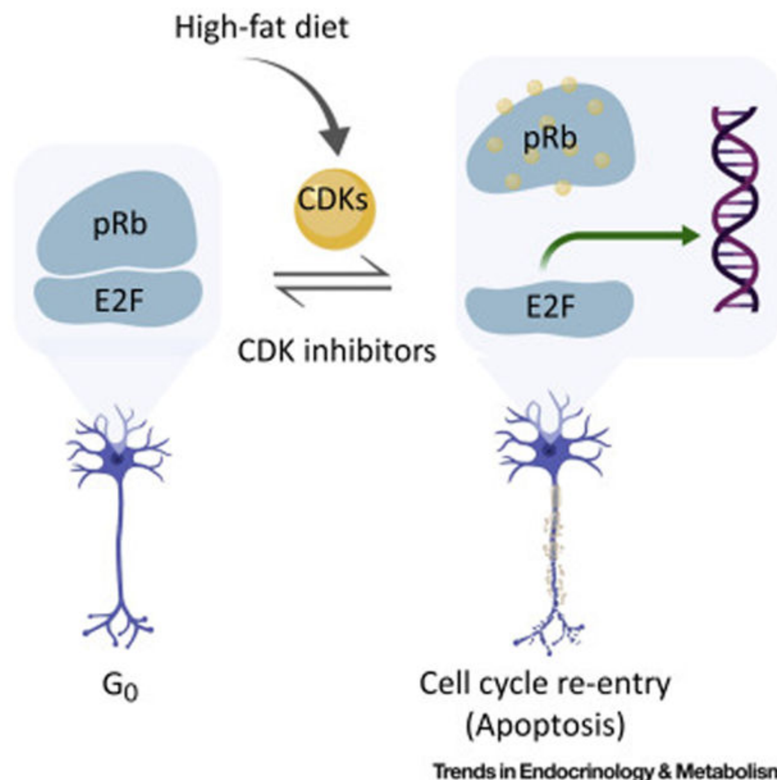

Figure 1. High Fat Feeding Triggers An E2F Transcriptional Program Leading to POMC Neuronal Dysfunction

Mature neurons are mitotically quiescent, due to active repression of E2Fs by pRb. Phospho-inactivation of pRb by cyclin dependent kinases (CDK2/CDK4/CDK6) releases E2Fs to find their target genes and initiates cell cycle re-entry. Neurons in most cases appear to be intolerant of expression of some E2F target genes and become dysfunctional. The pRb in hypothalamic POMC neurons is susceptible to CDK-mediated phospho-inactivation, leading to hyperphagia and obesity. Inhibition of CDKs by CDK inhibitors, such as abemaciclib, can reverse this process. In the case of complete pRb absence, POMC neurons (as well as many other neuron types) undergo cell death. (This figure was created using BioRender (https://biorender.com/).