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Relationship of arousal to circadian anticipatory behavior: Ventromedial hypothalamus - One Node in a Hunger/Arousal Network

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Abstract

The mechanisms by which animals adapt to an ever-changing environment have long fascinated scientists. Different forces, conveying information regarding various aspects of the internal and external environment, interact with each other to modulate behavioral arousal. These forces can act in concert or, at times, in opposite directions. These signals eventually converge and are integrated to influence a common arousal pathway, which, depending on all the information received from the environment, supports the activation of the most appropriate behavioral response. In this review we propose that the ventromedial hypothalamic nucleus (VMN) is part of the circuitry that controls food anticipation. It is the first nucleus activated when there is a change in the time of food availability, silencing of VMN ghrelin receptors decrease food anticipatory activity (FAA), and although lesions of the VMN do not abolish FAA, parts of the response are often altered. In proposing this model, it is not our intention to exclude parallel, redundant and possibly interacting, pathways that may ultimately communicate with, or work in concert with, the proposed network; but rather to describe the neuroanatomical requirements for this circuit, and illustrate how the ventromedial nucleus of the hypothalamus is strategically placed and connected to mediate this complex behavioral adaptation.

Keywords

ventromedial hypothalamus; food anticipatory activity; generalized arousal; locomotor activity

Introduction

Generalized arousal is thought of as a primitive, elementary function of the hindbrain reticular formation that is essential for the activation of any behavior (Pfaff, 2005). It synergizes with specific forms of arousal, such as those arising from sexual needs, thirst, fear and anger. Here we consider the role of hunger-stimulated arousal under experimental circumstances in which this force for arousal is set up against a normal circadian rhythm of arousal; that is, forcing the animal to be active for feeding during the light period, a time when circadian timing mechanisms normally mandate that a nocturnal animal be resting.

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Living in an environment where resources constantly fluctuate, be it on a daily, monthly, or annual basis, has provided certain challenges to terrestrial organisms. Homeostatic mechanisms have evolved that detect these changes and elicit compensatory rebounds to these challenges. Certain set-points are pre-determined and the body has antagonistic pathways that finely coordinate its responses to maintain them as close to this optimal value as possible, at all times. These challenges include hunger, thirst and sleep deprivation, to name a few. In addition to these homeostatically regulated variables, our responses are also driven by a circadian component, a rhythm that oscillates with a periodicity of approximately 24 h. How and where these forces interact and become integrated to elicit the most appropriate behavioral response remains poorly understood.

In terms of sleep physiology, it is well understood that there is a circadian (process C) and a homeostatic (process S) component that together determine the timing and amounts of sleep. The influence of each of these forces can be experimentally manipulated and Alexander Borbély and colleagues in Zurich have developed sophisticated mathematical models to predict sleep responses to variations in these two parameters (Borbély, 1982).

The circadian pacemaker is known to reside in the suprachiasmatic nuclei (SCN), located in the anterior hypothalamus. Lesioning these nuclei abolishes the consolidated periods of sleep and arousal, without affecting total sleep time (Ibuka *et al.*, 1980). In addition, SCN-lesioned animals exhibit uncompromised responses to sleep deprivation, suggesting that the two processes (S and C) are independent of each other, and furthermore, that the brain site where homeostatic sleep mechanisms reside is not within the SCN (Mistlberger *et al.*, 1983; Tobler *et al.*, 1983).

Like sleep, to date, no single brain region has been identified that is sufficient and necessary to generate food anticipatory activity. That is, destruction of any one nucleus, of the likely targets already identify by neuronal activation studies, has failed to completely or permanently abolish all food anticipatory behaviors. The dorsomedial nucleus of the hypothalamus (DMH) has attracted a fair amount of interest and speculation as the potential location for where the food entrainable oscillator may reside. The data supporting this theory come from the studies of Saper *et al.* showing that DMH-lesioned mice fail to anticipate a timed meal (Gooley *et al.*, 2006) and this group has also recently shown that restoration of Bmal1 signaling in the DMH using a viral vector is capable of rescuing FAA in this normally compromised transgenic model (Bmal1 knockout) (Fuller *et al.*, 2008). Collectively these findings would strongly support the hypothesis that indeed the location for the food entrainable oscillator is the DMH and that it relies on well-characterized transcription and translational circadian loops; however, numerous attempts by several independent laboratories have failed to replicate these findings (Landry *et al.*, 2006; Landry *et al.*, 2007; Mistlberger *et al.*, 2008; Moriya *et al.*, 2009; Pendergast *et al.*, 2009; Storch & Weitz, 2009) (for review see Mistlberger *et al.*, 2009).

The search for the location of the food-entrainable oscillator (FEO) continues to this day. Since FAA involves activity, eating, learning/conditioning, reward/reinforcement it was thought to lie within the central nervous system. Recently, FEOs have also been sought in peripheral organs such as the liver, kidney, heart, pancreas and stomach (Comperatore & Stephan, 1990; Davidson & Stephan, 1998; Damiola *et al.*, 2000; Yamazaki *et al.*, 2000; Davidson *et al.*, 2003). Interestingly, Davidson *et al.* have shown that peripheral tissues (e.g. liver, colon, stomach) express circadian genes products in a 24-h rhythmic cycle, and when food availability is restricted to daytime periods in nocturnal rodents, the phase of peripheral clock gene expression is set to the new feeding time (Damiola *et al.*, 2000; Yamazaki *et al.*, 2000; Davidson *et al.*, 2003). However, the phase of Per1 clock remained nocturnal during

subsequent food deprivation and during a 2-meal food restriction paradigm, which is not consistent with a circadian pacemaker (Davidson et al., 2003).

Redundant pathways control food anticipation

Given their critical role for survival, it is likely that redundant pathways have evolved to safeguard proper responses to changes in these variables, both in terms of detecting these changes, as well as executing the most appropriate behavioral response. This likely explains the inability of small localized brain lesions to abolish a behavioral response to sleep deprivation or hunger (timed meal). Although, if the lesion affects part(s) of the food anticipatory response, or if the region is activated at the time of food anticipation, the area is likely part of this redundant circuit.

We hypothesize that the increased arousal to a homeostatic challenge, such as food, is likely mediated by interactions among multiple brain networks, ultimately converging on common neuronal outputs that execute the most appropriate behavioral response to that particular challenge. As such, deficiencies in communication or, in extreme cases, total loss of one component of this system will not have catastrophic results for the organism. Knowing the neuroanatomical substrates that comprise the inputs (humoral), the integration of signals and neuroanatomical outputs (motor) of FAA is essential to understand the regulation of this circuit. One way of studying homeostatically driven mechanisms is by identifying neuronal groups that are selectively activated in response to stimulation of the homeostatic drive, e.g. sleep deprivation, hunger, thirst. This approach has, in fact, been extensively used and has yielded very fruitful studies correlating homeostatic need, e.g. following sleep deprivation, with selective activation of particular neuronal populations, e.g. the ventrolateral preoptic nucleus (Sherin *et al.*, 1996). This knowledge proved critical in establishing a network of interacting brain pathways that are hypothesized to ultimately determine vigilance state. This approach has also been used to study increases in motor activity in anticipation of food (Angeles-Castellanos *et al.*, 2004; Mendoza *et al.*, 2005; Meynard *et al.*, 2005; Johnstone *et al.*, 2006; Angeles-Castellanos *et al.*, 2007; Escobar *et al.*, 2007; Ribeiro *et al.*, 2007; Poulin & Timofeeva, 2008). Many brain regions have been shown to become activated accompanying feeding times, both before, during feeding or after feeding, when animals are subjected to either timed restrictive feeding, hypocaloric or hypoproteic diets (Feillet *et al.*, 2006). Our focus here is on the neuroanatomical structures likely involved in modifying behavior in response to these changes in the timing of resource availability, where in the brain these signals are first perceived and on the neurochemical substrates that likely communicate these signals to the brain.

The ventromedial hypothalamic nucleus as part of the FAA circuitry

We found that the VMN is the only brain region, of 16 brain nuclei reported to mediate changes in arousal, to become active when FAA is first manifested in response to food restriction at a unfavorable circadian time, compared to animals anticipating food delivered during the normal active (dark) period (Ribeiro *et al.*, 2007). Essentially, we entrained mice to anticipate a restricted meal whose timing coincided with lights off. After the animals were well entrained to the dark onset feeding time, we shifted the food presentation time of half of the mice to the middle of the light period, thus setting up these two forces for arousal – the new feeding time and the circadian light/dark cycle - against each other and monitored running wheel activity (Ribeiro *et al.*, 2007). The day after increased running wheel activity was first observed preceding the shifted food presentation time, matched pairs of animals (a shifted and a non-shifted control) were sacrificed at the same time during the anticipatory period and the level of neuronal activation - as judged by the expression of c-FOS protein - was compared between the animal expecting a meal at dark onset (non-shifted group) and

the animal expecting a meal in the middle of the light period (shifted group) (Ribeiro *et al.*, 2007). Of the brain regions examined, only the VMN had increased c-FOS expression in the shifted animals (Ribeiro *et al.*, 2007). This is not to say that had longer periods passed from when the FAA paradigm was imposed, other brain regions might not likewise have become activated. For instance, other neuronal activation studies have looked at c-FOS activation under various feeding schedules once FAA is robustly expressed (between 10 days and 3 weeks) (Angeles-Castellanos *et al.*, 2004; Mendoza *et al.*, 2005; Meynard *et al.*, 2005; Angeles-Castellanos *et al.*, 2007; Johnstone *et al.*, 2006; Poulin & Timofeeva, 2008), protocols which are very different from our study that aimed to identify the very first brain region to sense the changes in food availability time (hence we sacrificed the mice as close to the development of the behavioral response as possible). Likewise, we examined neuronal activation just before food presentation (0–1h before food presentation), it is therefore possible that other brain regions had been activated earlier and/or later than the VMN, even at the early stage of the development of FAA.

Other studies also suggest a role for the VMN in the circuitry controlling food anticipation. Although lesion experiments have demonstrated that no brain region alone, including the VMN, is required for the maintenance of FAA, lesions of the VMN did abolish anticipatory activity and corticosterone rhythms in 3 studies (Krieger, 1980; Inouye, 1982; Saito *et al.*, 1982) and in other studies attenuated FAA (Mistlberger & Rechtschaffen, 1985) and corticosterone rhythms (Honma *et al.*, 1987) in the first weeks after surgery, although the rhythms reappeared later. VMN disruption with colchicine has also been shown to disrupt restricted feeding-induced corticosterone rhythm (Choi *et al.*, 1998). Silencing ghrelin receptors within the VMH also decreases the amount of FAA (see below). The VMN is the only basomedial hypothalamic nucleus to be activated 2 hrs after arousal from hibernation in jerboas (El Ouezzani et al., 1999) and is essential to allow shifting of locomotor activity during restricted feeding (Challet et al., 1997). It is also important to point out that in these experiments, or in the theory proposed in this review, it is not possible to ascertain whether the VMN contains food entrainable oscillators but rather that it is part of a brain circuitry involved in food anticipation.

Feeding-related information is perceived by a combination of peripheral and central sensing mechanisms. In the periphery, gastrointestinal hormones are released by the enteric endocrine system, in response to either satiety (e.g. cholecystokinin, leptin) or hunger (e.g. ghrelin), establishing a finely-tuned homeostatic regulatory loop. Food anticipatory signals are likely communicated to the central nervous system by humoral factors since disrupting vagal or spinal afferent communication does not disrupt the ability of animals to anticipate food (Comperatore & Stephan, 1990; Davidson & Stephan, 1998, Davidson et *al.*, 2003), or these could be redundant pathways. We next examine the inputs and outputs that may underlie the mechanisms by which the VMN is involved in food anticipation.

Humoral Inputs to VMN

Even though the VMN has long been considered the “satiety center” of the brain, it is now known that it is a heterogeneous nucleus and activation or inhibition of neuronal subpopulations can either stimulate or inhibit feeding.

Energy balance and body weight are under the fine control of a variety of factors, produced by the gastrointestinal tract, which communicate to the brain via neural (Obici, 2009) and endocrine pathways (Grün & Blumberg, 2009). These molecules act on neurons in the arcuate nucleus, which is composed of two major neuronal subtypes involved in the regulation of food intake. Some arcuate neurons stimulate feeding and contain neuropeptide Y (NYP) and agouti-related peptide (AGRP), while others suppress feeding and contain proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript. Arcuate

neurons are responsive to peripheral hormonal signals that can activate or inhibit these opposing neuronal subtypes. Interestingly, POMC neurons have been shown to receive strong excitatory inputs from the ventromedial VMN, whereas NPY neurons receive only inhibitory inputs and predominantly from other arcuate neurons (Sternson *et al.*, 2005). Under conditions of starvation, the amount of excitatory input to POMC neurons is diminished, establishing a way for VMN neurons to control the activity of arcuate neurons and eventually increase food intake (Sternson *et al.*, 2005). The levels of circulating hormones (satiety-promoting: *e.g.* leptin and other postprandial gut peptides, hunger-promoting: *e.g.* ghrelin and PYY) influence the activity of these subpopulations of arcuate and VMN neurons, that will increase or decrease feeding according to short- and long-term signals of nutritional status.

The VMN is comprised of glucose-sensing neurons (Oomura *et al.*, 1964; Ono *et al.*, 1982; Ashford *et al.*, 1990; Mobbs *et al.*, 2001; Song & Routh, 2005), and VMN neurons have the ability to respond to a variety of humoral factors, including those that inhibit food intake: leptin (Dhillon *et al.*, 2006; Canabal *et al.*, 2007), cholecystokinin (Barnes *et al.*, 1991), insulin (Canabal *et al.*, 2007), alpha-melanocyte-stimulating hormone (Fu & van den Pol, 2008), histamine (Zhou *et al.*, 2007), glucagon-like peptide-1 (Sanz *et al.*, 2007), polypeptide YY (Huang *et al.*, 2008), estrogens (Zhou *et al.*, 2007), serotonin (Hikiji *et al.*, 2004); and factors that promote food intake: ghrelin (Chen *et al.*, 2005; Yanagida *et al.*, 2008), NPY (Davidowa *et al.*, 2002) and AGRP (Fu *et al.*, 2008), orexins (Muroya *et al.*, 2004), dopamine (Davidowa *et al.*, 2002) and norepinephrine (Lee *et al.*, 2007).

We hypothesize that, together, these properties of VMN neurons enable the brain to sense the body's metabolic state, and by using its many projections to arousal-promoting regions, the CNS can then mount the most appropriate behavioral response (favoring either energy conservation or energy dissipation, depending on the situation).

As this article covers the role of the VMN on arousal in anticipation of food presentation, we will focus on humoral inputs that promote arousal, hunger and/or feeding. Some of the orexigenic signals are produced peripherally. Among those, ghrelin is a primary candidate in the appearance of anticipatory behavior. Ghrelin is secreted in anticipation of food and signals meal time (Drazen *et al.*, 2006). Drazen et al. have suggested that ghrelin secretion is regulated by learned anticipation of regularly timed meals independent of the state of food deprivation making it a good candidate for "the regulation of anticipatory processes involved in food intake" (Drazen *et al.*, 2006). Our recent studies show that transgenic animals lacking ghrelin receptors have a much reduced FAA response, compared to their littermate controls (~50% reduction in FAA), and that oxyntic cells in the stomach change their expression of circadian genes to match the time of the feeding and not the light/dark cycle (LeSauter *et al.*, 2009). Additionally, bilateral silencing of ghrelin receptors specifically in VMN neurons using viral vector delivery of shRNA against growth hormone secretagogue 1a, likewise compromises the ability of these animals to properly anticipate a timed meal (~50% reduction in FAA) presented at either dark onset of during the middle of the light period, compared to control-injected mice (Ribeiro, LeSauter, Musatov, Arrieta-Cruz, Silver and Pfaff, *unpublished observations*). Together these studies provide strong evidence that ghrelin receptors in VMN neurons are involved in food anticipatory responses.

VMN neurons respond to gastric distension (Sun *et al.*, 2006), presumably through vagal afferents (Liu *et al.*, 2004), which suggest a neural pathway from the gut to the VMN. Ghrelin receptors are found in vagal afferents, and intravenous ghrelin administration decreases vagal electrical activity (Date *et al.*, 2002). There are, however, conflicting results on whether or not the vagus nerve is necessary for ghrelin-induced feeding (Date *et al.*, 2002; Date *et al.*, 2005; Arnold *et al.*, 2006).

Circulating gut peptides may also reach the VMN by crossing the blood-brain barrier or via circumventricular organs and act directly or indirectly on VMN neurons. The main effect of ghrelin on promoting food intake is thought to be through the NPY-AGRP neurons of the arcuate nucleus. Arcuate nucleus neurons bear growth hormone secretagogue receptors (the ligands for ghrelin) and expresses FOS following ghrelin administration (Howard *et al.*, 1996; Guan *et al.*, 1997; Yokote *et al.*, 1998; Mitchell *et al.*, 2001; Wang *et al.*, 2002; Zigman *et al.*, 2006). AGRP and NPY double knock-out mice do not exhibit ghrelin-induced feeding (Chen *et al.*, 2004). The VMN contains NPY and melanocortin 4 receptor (MC4R) receptors (Bouali *et al.*, 1995b; Lopez-Valpuesta *et al.*, 1996; Harrold *et al.*, 1999; Wisialowski *et al.*, 2000; Li & Ritter, 2004; Kishi *et al.*, 2005), suggesting that both NPY and POMC neurons project from the arcuate to the VMN. Administration of NPY and NPY agonist into the VMN increase feeding (Bouali *et al.*, 1995b). It is thought that peripheral ghrelin activates NPY/AGRP neurons inducing the release of these orexigenic peptides in their target regions, including the VMN.

Ghrelin, also, may act directly on the VMN by passing through the blood brain barrier or by diffusion from the arcuate nucleus region (Figure 1). The VMN contains growth hormone secretagogue receptors (Howard *et al.*, 1996;Guan *et al.*, 1997;Yokote *et al.*, 1998;Mitchell *et al.*, 2001;Zigman *et al.*, 2006), and ghrelin increases the firing rate in a large proportion (~65%) of VMN neurons in young rats (Yanagida *et al.*, 2008). In a study differentiating glucose-sensitive from other VMN cells, ghrelin increased firing rate in 40% of non-glucose-sensitive and decreased firing rate in 82% of glucose-sensitive VMN cells, inhibiting their anorexic effects and promoting food intake. In addition, ghrelin acts in VMH through fatty acid metabolism as central inactivation of AMP-activated protein kinase in the VMH blocks the orexigenic effect of ghrelin (Lopez *et al.*, 2008).

NPY and AGRP are secreted by neural afferents from the arcuate nucleus to several hypothalamic nuclei. As stated above, there are NPY receptors in the VMN, and NPY administration in the VMN stimulates food intake (Bouali *et al.*, 1995a). AGRP also stimulates feeding (Rossi *et al.*, 1998). It was thought to act as a melanocortin receptor antagonist in the VMN, but this effect has been questioned (Fu & van den Pol, 2008).

Orexins also act on the VMN and likely mediate increased arousal in response to hunger. Intracerebroventricular (i.c.v.) injection of orexins induces a rapid increase in food intake (Lubkin & Stricker-Krongrad, 1998; Sakurai *et al.*, 1998; Edwards *et al.*, 1999; Haynes *et al.*, 2000; Jain *et al.*, 2000; Sartin *et al.*, 2001). In addition to other hypothalamic nuclei, orexin receptors are present in the VMN (Trivedi *et al.*, 1998; Lu *et al.*, 2000; Hervieu et al., 2001; Cluderay *et al.*, 2002; Zhang *et al.*, 2005). Orexin-A and -B can activate NPY and inhibit POMC neurons in the arcuate and inhibit glucose-sensitive neurons in the VMN to stimulate food intake (Muroya *et al.*, 2004). I.c.v. injection of orexins also evokes emotion-related behavior such as stereotypy and hyperlocomotion (Ida *et al.*, 1999; Nakamura *et al.*, 2000).

Given their critical roles in mediating feeding and locomotor activities, several studies have examined the effects of orexins on FAA. Lack of orexin signaling has been shown to compromise the ability of animals to properly anticipate a timed meal, suggesting that orexin neurons could serve as an important efferent signal to locomotor areas (Mieda *et al.*, 2004). On the other hand, saporin ablation of hypocretin-2 cells in the lateral hypothalamus does not abolish FAA (Mistlberger *et al.*, 2003), orexin knockout animals, despite having reduced FAA, do have robust anticipatory rhythms of body temperature, suggesting, again, that orexins may be involved in an efferent pathway to induce locomotor activity, and the generation of FAA (Kaur *et al.*, 2008).

There are other putative FAA signals. For example intestinal apolipoprotein A-IV is rhythmically released and the rhythm is controlled by the timing of meals, with levels increasing before meal onset. Adrenal corticosterone levels are also rhythmic and peak in anticipation of meals (Shen *et al.*, 2005) and these levels are influenced by timing of meals (Wilkinson *et al.*, 1979; Honma *et al.*, 1984). During restricted feeding, corticosterone levels increase about 1 hr before food presentation. This precedes the premeal increase in apolipoprotein A-IV (Shen *et al.*, 2005). Adrenalectomy abolishes the rhythms of both corticosterone and apolipoprotein A-IV. These effects may take place at least in part in the VMH as adrenalectomy abolishes NPY induced insulin release and reduce the levels of Y1 and Y5 receptor mRNA in the VMN (Wisialowski *et al.*, 2000).

VMN arousal-promoting outputs

In addition to receiving massive humoral inputs conveying information about energy status of the organism, and the ability of VMN neurons to sense changes in glucose availability (Oomura *et al.*, 1964; Ono *et al.*, 1982; Ashford *et al.*, 1990; Mobbs *et al.*, 2001; Song & Routh, 2005) and modify their firing properties accordingly, the VMN must also faithfully signal this information to the appropriate brain regions to increase foraging and locomotion (Figure 1).

VMN has long been associated with feeding and the regulation of energy balance, starting from Hetherington and Ranson, who in 1940, first demonstrated that lesions at “the base of the diencephalon” (corresponding to the location of the VMN) were associated with extreme obesity in animals and humans (Hetherington & Ranson, 1940). Since then, many studies have examined the role of the VMN in energy homeostasis and collectively they have found that lesions (Hetherington & Ranson, 1940; Shimizu *et al.*, 1987), chemical blockade (Maes, 1980) or genetic ablation (Majdic *et al.*, 2002) of VMN neurons leads to obesity, while chemical (Shimazu & Ishikawa, 1981) or electrical (Stenger *et al.*, 1991) stimulation of this nucleus leads to satiety and decreased food intake. Position emission tomography shows blood flow increase of the hypothalamic nuclei during hunger and a decrease after eating (Tataranni *et al.*, 1999; Morris & Dolan, 2001). Functional magnetic resonance imaging shows decrease in signal after eating in PVN and VMN (Matsuda *et al.*, 1999). Interestingly, Kokoeva et al. recently demonstrated that changes in energy balance are accompanied by neurogenesis in VMN (Kokoeva *et al.*, 2005), thus illustrating the dynamic changes that can occur within hypothalamic feeding circuits depending on the metabolic state of the animal.

Morphologically, the VMN can be subdivided into 3 major cytoarchitectural areas: the ventrolateral region, a central region and the dorsomedial region; and these three areas are anatomically, neurochemically and behaviorally distinct. Each of these regions has distinct projection patterns (Saper *et al.*, 1976; Canteras *et al.*, 1994), and they can broadly be grouped as ascending fibers that innervate anterior targets, including medial and lateral preoptic regions, lateral septum, preoptic and thalamic periventricular nuclei, bed nucleus of the stria terminalis and the amygdala (Saper *et al.*, 1976; Krieger *et al.*, 1979; Canteras *et al.*, 1994). The majority of these long projections arise from the ventrolateral VMN (Conrad & Pfaff, 1976; Saper *et al.*, 1976; Krieger *et al.*, 1979; Canteras *et al.*, 1994), although some sparse dorsomedial projections to the preoptic area (POA) have also been observed (Krieger *et al.*, 1979; Canteras *et al.*, 1994).

Particularly relevant to the promotion of arousal and locomotion are the projections to the POA, a well-characterized brain region known to be antidromically activated along with the midbrain locomotor region in promoting locomotor behaviors (Sakuma, 1976; Swanson *et al.*, 1987) (Figure 1).

The VMN also projects directly (Moga & Moore, 1997) or indirectly (Canteras *et al.*, 1994; Moga & Moore, 1997) to the SCN and can allow for phase shifts in locomotor activity when homeostatic challenges are set in antiphase to environmental light/dark conditions (Challet *et al.*, 1997). More rostrally, VMN projecting fibers extend to the cholinergic ventral and diagonal band of broca (Conrad & Pfaff, 1976; Krieger *et al.*, 1979), which are well known to contribute to the promotion of arousal and cortical activation (Jones, 2004).

VMN neurons also send dense excitatory projections to the nearby arcuate nucleus, specifically to the POMC neurons, and this excitatory tone is decreased with fasting (Sternson *et al.*, 2005), thus demonstrating the dynamic interplay between energy state and output strength from the VMN. Food restriction also leads to morphological changes of VMN neurons, shortening long primary dendrites of lateral projecting neurons in the ventrolateral VMN and reducing the soma area of dorsomedial VMN neurons with medially projecting long primary dendrites (Flanagan-Cato *et al.*, 2008).

Caudal projections from VMN generally follow three major routes: one pathway travels along the medial hypothalamus projects to the ventral tegmental area, the supramammillary nucleus and mammillary complex; a second pathway travels along the periventricular region and eventually projects to the posterior hypothalamus, including the histaminergic tuberomammillary nucelus, the noradrenergic locus coeruleus and the serotinergic dorsal raphe nuclei (Saper *et al.*, 1976; Krieger *et al.*, 1979) [all members of the classical ascending reticular activating system originally proposed by Moruzzi and Magoon in 1949 (Moruzzi & Magoun, 1949)]; and finally, a pathway that travels along the supraoptic commissure, which eventually projects to the zona incerta, central amygdala, peripeduncular nucleus and central tegmental fields (Conrad & Pfaff, 1976; Saper *et al.*, 1976; Krieger *et al.*, 1979; Canteras *et al.*, 1994).

The VMN also has extensive projections to the nearby arousal and foraging promoting orexinergic lateral hypothalamic fields (Arees & Mayer, 1967; Sutin & Eager, 1969; Millhouse, 1973) (Figure 1). These findings have recently been confirmed, and extended, and VMN does indeed send a large number of projections to all areas of lateral hypothalamus (LH), with particularly high density to the medial LH; in fact, 23% of all LH neurons receive VMN appositions (Yoshida *et al.*, 2006). Electrical stimulation of the LH has been shown to increase locomotor activity, and these increases are not mediated via the mibrain locomotor region (Sinnamon & Stopford, 1987).

Very dense VMN projections are likewise observed traveling up to the mesencephalic reticular formation, including the anterior ventromedial midbrain, posterodorsal midbrain, which have been previously shown to facilitate locomotor behavior in anesthetized rats (Sinnamon *et al.*, 1987) (Figure 1).

Finally, labeled projections from the VMN have been identified as far caudal as the pontine and mesencephalic reticular formation, including gigantocellular neurons (Conrad & Pfaff, 1976; Krieger *et al.*, 1979; Parent & Steriade, 1981; Canteras *et al.*, 1994), which we hypothesize play a key role in the modulation of a generalized arousal state (Martin, Pavlides and Pfaff, *unpublished observations*).

Promotion of Arousal/Locomotion: A theory of how FAA is initiated

We propose that activation of VMN neurons initiates FAA by virtue of its efferents to three neural groups: POA locomotor region, midbrain locomotor region and lateral hypothalamic orexin neurons (via glutamatergic projections) (Figure 1).

The most well characterized brain areas for promoting locomotor behaviors in the brain are the POA and the midbrain locomotor region. Both these centers receive dense innervations from the VMN (Conrad & Pfaff, 1976; Saper *et al.*, 1976; Krieger *et al.*, 1979; Canteras *et al.*, 1994). Sinnamon et al. have identified discrete locations within the POA where chemical or electrical stimulation leads to locomotor stepping in anesthetized rats (Sinnamon, 1987; Levy & Sinnamon, 1990; Sinnamon *et al.*, 1991; Sinnamon, 1992). Within the POA, the areas most likely to induce locomotor behaviors in anesthetized rats extend from the ventral bed nucleus of the stria terminalis, the lateral part of the POA, horizontal band of the diagonal band of Broca, anterior hypothalamic area and the medial and rostral parts of the ventral pallidum (Sinnamon, 1992). Likewise, the midbrain locomotor region is composed of discrete regions where electrical stimulation is particularly effective at initiating locomotion (Mori *et al.*, 1978), specifically the medioventral medulla, including the nucleus reticularis gigantocellularis and the nucleus reticularis ventralis that are essential for locomotion (Garcia-Rill *et al.*, 1987).

The POA locomotor region and the midbrain locomotor region have been shown to communicate with each other; in fact, using a combination of neuroanatomical tracings and electrophysiological recordings Swanson et al. have established that the POA and the midbrain locomotor region (including the zona incerta and pedunculopontine tegmental area) exhibit bidirectional connectivity, and they also demonstrated that neurons in the POA are antidromically activated with stimulation of the midbrain locomotor region, and vice-versa (Swanson *et al.*, 1987) (Figure 1).

Neurons within the lateral hypothalamus are also involved in locomotor behaviors, for instance animals bred for high running wheel activity have higher c-FOS-positive neurons within the lateral hypothalamus compared to animals whose wheels were locked (Rhodes *et al.*, 2003) (Figure 1). Furthermore, electrical stimulation of the lateral hypothalamus elicited locomotor activity in rats (Sinnamon *et al.*, 1987), central administration of orexins increases locomotor activity (Nakamura *et al.*, 2000) and cerebrospinal levels of orexin-A is increased after forced activity (Martins *et al.*, 2004). Furthermore, Yamanaka et al. reported that levels of orexin mRNA in lateral hypothalamic neurons **are** inversely correlated to blood glucose levels, leptin and food intake (Yamanaka *et al.*, 2003). Animals that lack orexin neurons fail to increase their locomotor activity and arousal responses under fasting conditions (Yamanaka *et al.*, 2003). VMN neurons send dense projections to LH neurons (Arees & Mayer, 1967; Sutin & Eager, 1969; Millhouse, 1973; Conrad & Pfaff, 1976; Saper *et al.*, 1976), which are hypothesized to be excitatory, glutamatergic, in nature, that presumably activate food-seeking mechanisms.

Future directions

Given its vast numbers of humoral inputs, the ability of its neurons to sense and respond to changes in glucose levels, and its connectivity to major arousal promoting areas, we propose that the VMN is a prime candidate for the integration of feeding signals and for orchestrating the most appropriate behavioral response to constant fluctuations in resource availability.

Our previous work (Ribeiro *et al.*, 2007) identified VMN neurons as first responders in an FAA paradigm. Silencing VMH ghrelin receptors reduces FAA. . Now we must identify the transcriptomes of those neurons, first by immunocytochemistry and then by single cell RT/PCR. For example,, using a combination of retrograde markers microinjected amongst orexin neurons and RT/PCR, we will be able to test the hypothesis that glutamatergic VMN neurons have the correct connectivity to excite orexin neurons, thus to increase the animal's arousal prior to food availability. Subsequent electron microscopy of orexin neurons

following anterograde marker injections in VMN would confirm that synapses from those VMN neurons are actually made onto immunochemically-identified orexin neurons, which have previously been implicated in increasing locomotor activity during FAA (Mieda *et al.*, 2004; Kaur *et al.*, 2008).

Additionally, future studies aimed at investigating the possibility that VMN neurons could act as food entrainable oscillators should be undertaken. For instance, *in vivo* recording of VMN neurons during food anticipatory activity, both during food restriction days, as well as *ad libitum* and food deprivation days (typical food anticipatory protocol) – presumably these neurons should increase their firing rate during times of food anticipation, the increased firing should cease under *ad libitum* food, and once food is scarce the firing of these neurons should increase to match the anticipatory timing prior to *ad libitum* conditions. Again, a complete transcriptome analysis of these electrophysiologically-identified neurons may reveal novel signaling peptides or receptors involved in anticipating temporally restricted feedings.

In conclusion, the evidence that VMN neurons are activated when food anticipatory activity is first expressed (Ribeiro *et al.*, 2007), that ghrelin may be a signal for food anticipation (LeSauter *et al.*, 2009) and silencing VMN ghrelin receptors reduces FAA (results from our lab), and that different types of VMN lesions either abolish or decrease at least some food anticipation responses (Krieger, 1980; Honma *et al.*, 1987; Inouye, 1982; Mistlberger & Rechtschaffen, 1985; Saito *et al.*, 1982), strongly suggest that the VMN is part of a redundant circuitry controlling FAA.

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Abbreviations

AGRP	agouti-related peptide
FAA	food anticipatory activity
FEO	food entrainable oscillator
i.c.v	intracerebroventricular
LH	lateral hypothamalus
MC4R	melanocortin 4 receptor
midbrain LOCO	midbrain locomotor regions
POA	preoptic area
POA LOCO	preoptic locomotor area
NPY	neuropeptide Y
POMC	proopiomelanocortin
SCN	suprachiasmatic nuclei
VMN	ventromedial nucleus of the hypothalamus

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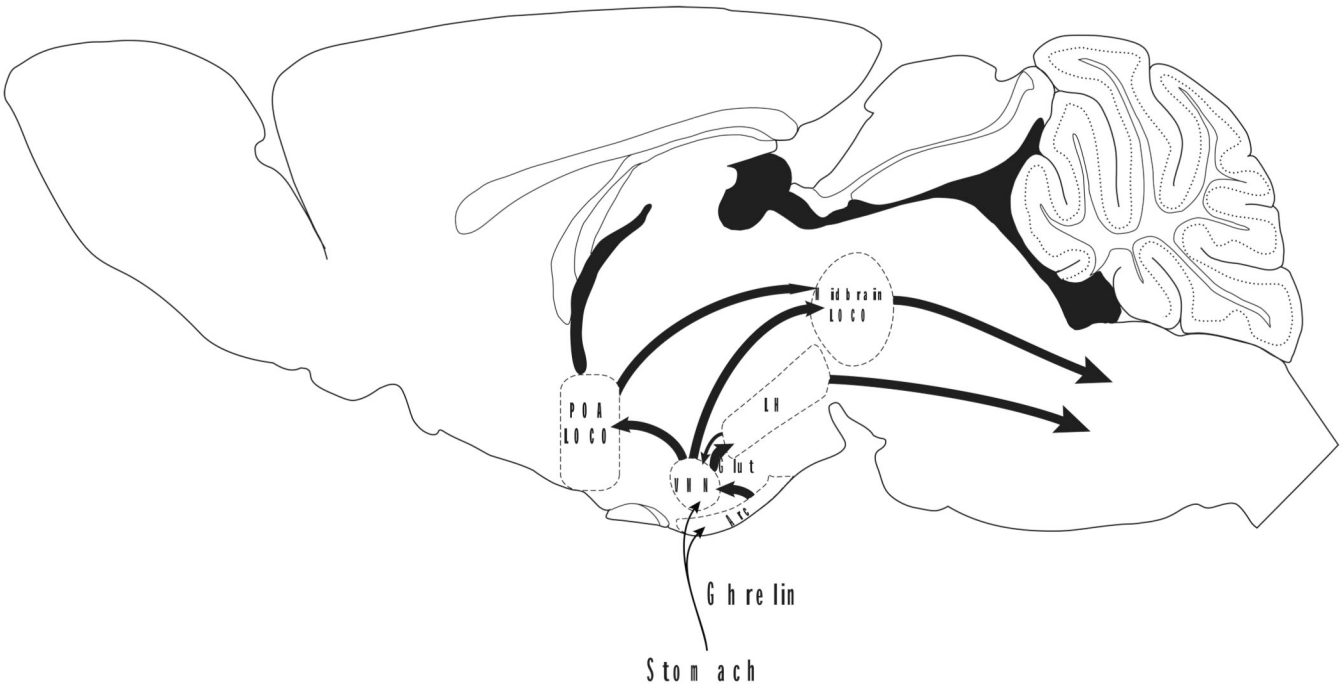

Figure 1.

Activation of arousal by hunger: Minimally sufficient model to explain how ventral medial hypothalamic (VMN) neurons drive food anticipatory activity. Schematic representation of humoral inputs to VMN; and neuroanatomical projections from VMN to brain regions involved in increasing generalized arousal in response to hunger (Only well-established neuroanatomical connections are depicted.) VMN: ventromedial nucleus of the hypothalamus; POA LOCO: preoptic locomotor region; LH: lateral hypothalamus (particularly orexin-containing neurons); Midbrain LOCO: midbrain locomotor region, Arc: arcuate nucleus, Glut: glutamatergic projections. Figure adapted from Paxinos and Franklin (Paxinos & Franklin, 2001).