BRAIN NUTRIENT SENSING IN THE CONTROL OF ENERGY BALANCE: NEW INSIGHTS AND PERSPECTIVES

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BRAIN NUTRIENT SENSING IN THE CONTROL OF ENERGY BALANCE: NEW INSIGHTS AND PERSPECTIVES

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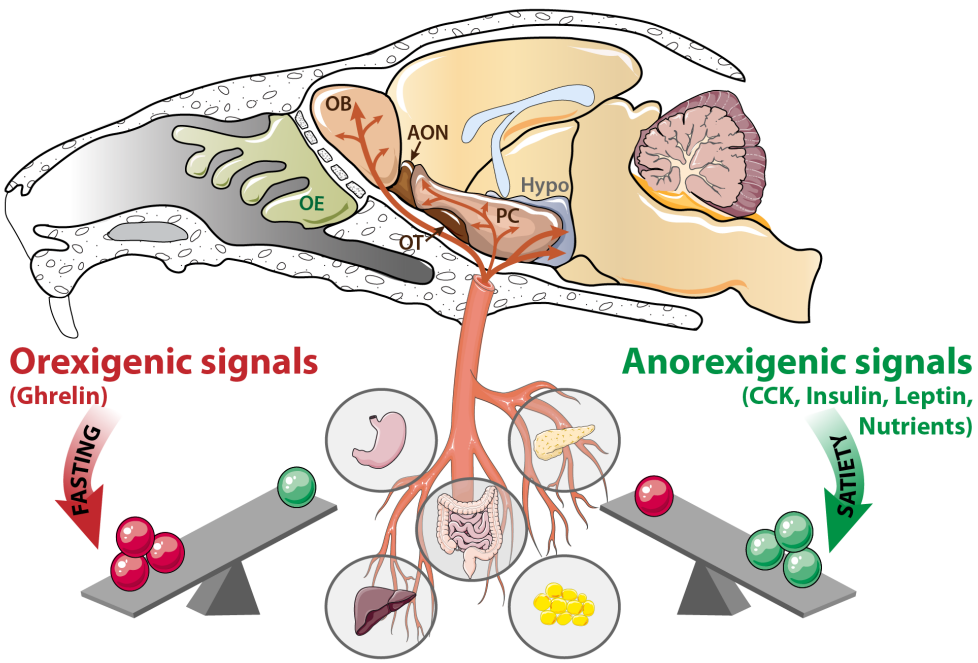

The olfactory system is a metabolic sensor like the hypothalamus. According to the nutritional status, a balance exists between peripheral signals delivered by the stomach, intestine, liver, pancreas, and adipose tissue. During fasting, orexigenic signals (ghrelin, and nutrients scarcity) prevail. In contrast, during satiation, anorexigenic signals (CCK, insulin, leptin and nutrients abundance) are predominant. These signaling molecules reach the central nervous system via the blood flow, where they target the hypothalamus (Hypo) as well as a variety of olfactory structures: OE, olfactory epithelium; OB, olfactory bulb; AON, anterior olfactory nucleus, OT, olfactory tubercle; PC: piriform cortex; CCK, cholecystokinin.
Image: Figure 1 from Julliard et al. 2017.

Julliard A-K, Al Koborssy D, Fadool DA and Palouzier-Paulignan B (2017) Nutrient Sensing: Another Chemosensitivity of the Olfactory System. *Front. Physiol*. 8:468. doi: 10.3389/fphys.2017.00468

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The present Research Topic proposes reviews and original articles covering the complex field of study of the importance of brain nutrient-sensing in the control of energy balance. It aims to give a clear view on the current questions addressed by the scientific community, and to raise new questions to achieve a full understanding of the impact of nutrient-sensing, and more generally nutrition, on brain functions.

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EDITORIAL
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Editorial: Brain Nutrient Sensing in the Control of Energy Balance: New Insights and Perspectives

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Editorial on the Research Topic

Brain Nutrient Sensing in the Control of Energy Balance: New Insights and Perspectives

In the past decade the research on the homeostatic or non-homeostatic control of energy balance by the brain nutrient sensing has seen a profound evolution, and understanding the cellular and molecular mechanisms by which circulating nutrients act on brain have become a highly competitive field, with outcomes leading to several strategies to prevent or treat obesity and relative disorders. While studies on the role of hypothalamic networks have been the most numerous, more and more studies have recently described the crucial roles of other brain networks in the homeostatic and non-homeostatic control of energy metabolism. The present research topic proposes reviews and original articles covering this complex field of study of the importance of brain nutrient-sensing in the control of energy balance.

Glucose and lipids have been the most studied nutrient involved in brain nutrient-sensing. A Perspective Article with original data by Fioramonti et al. summarizes the recent advances in the cellular and molecular mechanisms of hypothalamic glucose detection. The authors pointed out the critical role of reactive oxygen species in glucose-sensing, and also discussed the newly discovered role of transient receptor potential canonical channels (TRPC) in glucose detection. In addition, they pointed out the possible implication of metabolism-independent glucose-sensing mechanisms through either the sodium-glucose cotransporter (SGLT) or sweet taste receptors. This article, thanks to original data, helped understanding the controversy around the direct glucose-sensing properties of the melanocortin neurons of the arcuate nucleus of the hypothalamus. Zhou et al. presented another Perspective Article, on the role of hypothalamic glucose-inhibited (GI) neurons in the detection and regulation of hypoglycaemia, which is a profound threat to the brain since glucose is its primary fuel. Their discussed the features of GI neurons in the ventromedial hypothalamus (VMH), which expresses neuronal nitric oxide synthase (nNOS), and in the perifornical hypothalamus (PFH) which expresses orexin. They then highlighted new interesting approaches to prevent hypoglycaemia: to enhance antioxidant systems (such as glutathione or thioredoxin), to explore how orexin-GI neurons play a role in stimulating food intake (which leads to a corrected glycemia), and the relationships between hypothalamic sensors and hindbrain neurocircuitry… Finally, an original article from Yuan et al. on extra-hypothalamic glucose-sensing networks demonstrated the role of Nesfatin-1 neurons in the lateral parabrachial nucleus (LPBN) in food intake and energy expenditure controls. This effect is mediated through the excitation of most of the glucose-inhibited neurons in the LPBN, which leads to enhanced UCP1 expression in brown adipose tissue via the melanocortin system.

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Two Review Articles treated different impact of lipid-sensing on either energy homeostasis or mood and cognitive functions. Larrieu and Layé addressed the question of a link between dietary omega-3 polyunsaturated fatty acids and neuropsychiatric diseases such as anxiety and depression. They discussed potential mechanisms involved in the neuroprotective and corrective activity of ω3 PUFAs in the brain, in particular the sensing activity of free fatty acid receptors, the activity of the PUFAs-derived endocannabinoid system and the hypothalamic-pituitary adrenal axis. Among lipids, several studies reviewed by Cruciani-Guglielmacci et al. suggested that ceramides could be involved in the regulation of energy balance in both hypothalamic and extra-hypothalamic areas. Interestingly, under lipotoxic conditions, these ceramides were shown to play a role in the dysregulation of central control of glucose homeostasis. Thus, pushing ceramide metabolism toward the synthesis of less harmful lipids, such as Sphingosine 1-phosphate, with the use of sphingosine kinase 1 activators could represent a new therapeutic approach to counteract lipotoxicity.

The impact of nutrients and energy status of olfactory functions and, reciprocally, the impact of this system on food intake is a fairly new field of investigation arousing the scientific committee. In the present topic, two original research articles and one review article highlighted the role of the olfactory bulb (OB) in the control of food intake and whole body metabolism. In particular Kovach et al. explored the role of the potassium channel Kv1.3, whose deletion in the OB lead to "super-smeller mice," on mitochondrial structure and glucose utilization. These changes are initiated at the OB level, but they could drive whole system changes in metabolism. Another team, Chelminski et al., questioned the role of leptin receptors in the OB, suggesting a role for leptin in odor-evoked activities. They showed that cellular dynamics in the OB of leptin-deficient mice (ob/ob) are deeply modified in the context of olfactory learning. The review by Julliard et al. emphasized the link between nutrient sensing and the olfactory system. They first recalled that orexigenic peptides such as ghrelin and orexin increase olfactory sensitivity, which in turn, is decreased by anorexigenic hormones such as insulin and leptin. Then, they summarized recent findings on nutrient sensing in the OB. This new line of investigation at the crossroad between olfaction and food behavior could contribute to better determining the etiology of metabolic disorders.

Finally, a review article by de Lucia et al. highlighted the emerging molecular pathways that govern the dietary regulation of neural stem cells (NSCs) during aging. They discussed the importance of the Sirtuin, mTOR and Insulin/Insulin like growth factor-1 pathways as well as the significant role played by epigenetics in the dietary regulation of NSCs. Taking all the data together, nutrition may be a promising mode of intervention to regulate NSCs and prevent the cognitive decline associated with aging.

Overall, the present topic, through original or perspective articles and reviews, gives a clear view on the current question addressed by the scientific community working on brain glucose-sensing, and opens to new questions which still need to be addressed to fully understand the impact of nutrient-sensing, and more generally nutrition, on brain functions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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PERSPECTIVE
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Recent Advances in the Cellular and Molecular Mechanisms of Hypothalamic Neuronal Glucose Detection

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The hypothalamus have been recognized for decades as one of the major brain centers for the control of energy homeostasis. This area contains specialized neurons able to detect changes in nutrients level. Among them, glucose-sensing neurons use glucose as a signaling molecule in addition to its fueling role. In this review we will describe the different sub-populations of glucose-sensing neurons present in the hypothalamus and highlight their nature in terms of neurotransmitter/neuropeptide expression. This review will particularly discuss whether pro-opiomelanocortin (POMC) neurons from the arcuate nucleus are directly glucose-sensing. In addition, recent observations in glucose-sensing suggest a subtle system with different mechanisms involved in the detection of changes in glucose level and their involvement in specific physiological functions. Several data point out the critical role of reactive oxygen species (ROS) and mitochondria dynamics in the detection of increased glucose. This review will also highlight that ATP-dependent potassium (K_{ATP}) channels are not the only channels mediating glucose-sensing and discuss the new role of transient receptor potential canonical channels (TRPC). We will discuss the recent advances in the determination of glucose-sensing machinery and propose potential line of research needed to further understand the regulation of brain glucose detection.

Keywords: glucose, hypothalamus, pro-opiomelanocortin neurons, transient receptor potential channels, reactive oxygen species, electrophysiology

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Glucose homeostasis needs to be highly regulated to maintain blood glucose level constant. This is mandatory avoid any drop in blood glucose level as this nutrient is the preferred fuel source for the brain. Furthermore, increased blood glucose level also needs to be controlled to prevent long-term complication of excessive energy supply. Detection of changes in glucose level is achieved by glucose-sensors which work in concert to maintain glucose homeostasis. They are located at several anatomical sites at the periphery including taste buds of the tongue, the carotid bodies, the intestine, the portal vein, and the endocrine pancreas. In the central nervous system these sensors, called glucose-sensing neurons, are also localized in different brain areas. These neurons have initially been characterized in the hypothalamus. Even if they seem enriched within different hypothalamic nuclei, they have also been found in other areas including the brainstem, the cortex,

the hippocampus, the thalamus, the amygdala, or the olfactory bulbs (see for review Steinbusch et al., 2015. Thus, glucose-sensing neurons have been suggested to be involved (1) in the control of feeding initiation and termination and (2) through the modulation of the autonomic nervous system, in functions modulated in response to changes in brain glucose levels among which pancreatic hormonal secretion, β-cell proliferation, thermogenesis, and hepatic glucose production. The presence of these neurons in extra-hypothalamic or brainstem structures suggests that they could be involved in other physiological functions than the control of energy homeostasis such as memory, olfaction, motivation, or reward for instance. However, characterization of physiological functions modulated by glucose-sensing needs further attention. In this review, we will discuss the recent advances made on the role of hypothalamic glucose-sensing neurons in the control of energy homeostasis.

CHARACTERIZATION OF HYPOTHALAMIC GLUCOSE-SENSING NEURONS

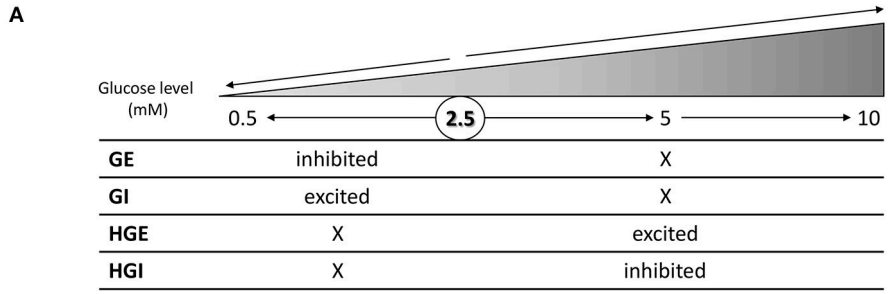
The idea that specialized cells could detect changes in glucose level originated from Oomura' and Anand's groups (Anand et al., 1964; Oomura et al., 1964). Later, Oomura and colleagues conclusively demonstrated the presence of specialized glucose-sensing neurons in showing that the direct electro-osmotic application of glucose altered the activity of hypothalamic neurons (Oomura et al., 1974). These neurons are now defined as cells able to adapt their electrical activity by directly detecting changes in extracellular glucose level. By definition, glucose-excited (GE) neurons increase their electrical activity when glucose level rises. By opposition, glucose-inhibited (GI) neurons increase their activity when glucose level decreases. It is important to note that glucose-sensing neurons directly detect changes in glucose level. In the brain, many neurons can see their electrical activity modulated by glucose but essentially due to pre-synaptic inputs coming from "true glucose-sensing" cells. Thus, in this review, we will refer as glucose-sensing cells, neurons which directly detect changes in glucose level.

Within the medio-basal hypothalamus, which contains both the arcuate (ARC) and ventromedian nuclei (VMN), four populations of glucose-sensing neurons have been characterized according to the changes in glucose level they detect. Evidence suggest that specialized glucose-sensing neurons detect changes in glucose level either above or below 2.5 mM (Fioramonti et al., 2004; Wang et al., 2004; Penicaud et al., 2006; Chretien et al., 2017; **Figure 1A**). These neurons are referred as GE or GI neurons (respectively inhibited or activated in response to decreased glucose level below 2.5 mM) and HGE or HGI neurons (for high-glucose-excited or -inhibited, respectively activated or inhibited by changes above 2.5 mM). Interestingly, the electrical activity of GE and GI neurons is only changed in response to glucose change below 2.5 mM and not altered by changes in glucose level above 2.5 mM (Fioramonti et al., 2004; Wang et al., 2004). Similarly, we found that HGE and HGI neurons only change their electrical activity in response to changes in glucose level above 2.5 mM but not below this level (Fioramonti et al., 2004). Finding these different sub-populations of glucose-sensing neurons enriched the debate on the glucose level present in the hypothalamus.

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; ARC, arcuate nucleus; BBB, blood-brain-barrier; DMN, dorsomedian nucleus; GE neurons, glucose-excited neurons; GI neurons, glucose-inhibited neurons; GHRH, growth hormone-releasing hormone; GLUT, glucose transporter; GnRH, gonadotrophin releasing hormone; HGE neurons, high-glucose excited neurons; HGI neurons, high-glucose inhibited neurons; LH, lateral hypothalamus; KATP channel, potassium ATP-dependent channelMBH, mediobasal hypothalamus; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; PO, preoptic area; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; ROS, reactive oxygen species; SF1, steroidogenic factor 1; SGLT, sodium-glucose cotransporters; TRPC, transient receptor potential canonical; VMN, ventromedial nucleus.

Brain Glucose Levels

The level of brain glucose is a process finely regulated. Glucose transporter 1 (GLUT1) seems to be the primary transporter controlling brain glucose entry (Cardoso et al., 2010). The high affinity of this transporter for glucose justifies the level found in the brain which is about 30% of the blood level and close to 2 mM at baseline (∼7–8 mM blood glucose). This is the case in the hypothalamus or other areas including the hippocampus and striatum for instance (McNay and Gold, 1999; McNay et al., 2001; de Vries et al., 2003; Dunn-Meynell et al., 2009; Langlet et al., 2013). Interestingly, hypothalamic glucose level does not seem to fluctuate significantly between meals (Dunn-Meynell et al., 2009) or even after fasting (Langlet et al., 2013). Langlet and colleagues even found that glucose level in the ARC is slightly increased after fasting due to an increased permeability of the blood-brain-barrier (BBB). Thus, only profound manipulations of blood glucose level would induce changes in hypothalamic levels including insulin-induced hypoglycemia or pathological hyperglycemia. Such conditions would make hypothalamic levels to fluctuate between 0.2 and 4.5 mM (see for review Routh et al., 2014). Nevertheless, the finding of specialized neurons able to detect changes above 2.5 or even 5 mM suggests that, in confined areas, glucose level could be increased closer to level found in the blood. This could be the case around fenestrated capillaries of the BBB present in the ventral part of the ARC (Ciofi, 2011; Langlet et al., 2013). In support of this hypothesis, HGE and HGI neurons have only been found in the ARC (Fioramonti et al., 2004) and not in the VMN (Song et al., 2001). In this later study, neurons activated by increased glucose level above 5 mM have been found in the VMN but due to pre-synaptic glutamate inputs and not though a direct detection (Song et al., 2001). Local changes of glucose level around fenestrated capillaries would not be detectable by the available techniques including the microdialysis which does not present sufficient spatial resolution to monitor changes in confined environments. Determining (1) whether HGE- and HGI-like neurons can be found in extra-hypothalamic circumventricular organs in which the BBB is fenestrated (e.g., area postrema of the hindbrain, the subfornical organ and the vascular organ of lamina terminalis); (2) the real concentration of glucose in these micro-environments, will help characterizing brain spots containing these specialized glucose-sensing neurons as well as their physiological functions.



B

PO	ARC				VMN		DMN		LH		PVN	
GE	GE	GI	HGE	HGI	GE	GI	GE	GI	GE	GI	GE	GI
GnRH	POMC ?	NPY GHRH	POMC ?	GHRH	SF1 ?	SF1 ?	GABA	GABA	MCH	orexin NPY GABA	Nesfatin-1	?

FIGURE 1 | Schematic overview of the changes in glucose level detected **(A)** and nature **(B)** of the four subtypes glucose-sensing neurons present in the hypothalamus. **(A)** GE and GI neurons are respectively inhibited and activated by changes in glucose level below 2.5 mM and are non-sensitive to change above 2.5 mM. By opposition, HGE and HGI neurons are respectively activated and inhibited by changes above 2.5 or 5 mM while their activity is not altered by changes below 2.5 mM. The symbol "X" means that the electrical activity is not changed in comparison to the basal activity at 2.5 mM. **(B)** Nature of hypothalamic glucose-sensing neurons. ARC, arcuate nucleus; DMN, dorsomedian nucleus; LH, lateral hypothalamus; PVN, paraventricular nucleus; PO, pre-optic area; VMN, ventromedian nucleus. The symbol "?" means that the nature of glucose-sensing neurons has yet to be determined.

NATURE OF HYPOTHALAMIC GLUCOSE-SENSING NEURONS

In order to better appreciate the physiological role of glucose-sensing neurons in the hypothalamus, several groups have dedicated means to determine the nature of these cells. Thus, studies have essentially tried to determine glucose-sensing properties of known neuropeptide-expressing neurons of the hypothalamus (**Figure 1B**). In the lateral hypothalamus (LH), orexin-, neuropeptide Y (NPY)-, and GABA-expressing neurons present GI-like properties whereas melanin-concentrating-hormone (MCH) neurons are GE-type (Williams et al., 2001; Burdakov et al., 2005; Gonzalez et al., 2008; Karnani and Burdakov, 2011; Marston et al., 2011; Sheng et al., 2014). In the paraventricular nucleus (PVN), some GE neurons have been shown to be nesfatin-1-expressing cells (Sedbazar et al., 2014). The identity of the remaining glucose-sensing neurons in this nucleus has yet to be determined. In the dorsomedian nucleus (DMN), some GABAergic neurons are GE or GI neurons (Otgon-Uul et al., 2016). In the VMN, GE or GI neurons have been shown to express the transcription factor Steroidogenic factor 1 (SF1; Toda et al., 2016). This would suggest that VMN glucose-sensing neurons are glutamatergic since most of SF1 neurons express this neurotransmitter (McClellan et al., 2006; Tong et al., 2007). This hypothesis is supported by the study of Tong et al. showing that the counter-regulation to hypoglycemia is impaired in mice lacking glutamate release in VMN SF1 neurons. Nevertheless, VMN GE or GI neurons could express other neurotransmitter or neuropeptide since glutamate is not the only transmitter expressed in this nucleus or even in SF1 neurons (McClellan et al., 2006). In the anterior part of the hypothalamus, GnRH (gonadotrophin releasing hormone) neurons of the preoptic area (PO) are GE (Roland and Moenter, 2011a,b; Beall et al., 2012). Interestingly, only GnRH neurons of this area seem to be glucose-sensing (Roland and Moenter, 2011b). In the ARC, we and others show that the large majority of GI neurons express NPY (Muroya et al., 1999; Fioramonti et al., 2007; Murphy et al., 2009). Stanley et al. also showed that ARC growth-hormone-releasing hormone (GHRH)-expressing neurons present GI- or HGI-like properties as they detect changes in the 0.2–10 or 4–10 mM windows level (Stanley et al., 2013). The nature of GE and HGE neurons is to debate as several studies show different results regarding their identity being POMC-expressing cells or not.

Are POMC Neurons Glucose-Sensing Cells?

Some studies using electrophysiology on brain slices suggested that POMC neurons could be either GE or HGE-type neurons as their activity is modulated by changes in extracellular glucose level (Ibrahim et al., 2003; Claret et al., 2007; Parton et al., 2007). Other studies including one from our lab did not find POMC neurons directly glucose-sensing (Wang et al., 2004; Fioramonti et al., 2007). Nevertheless, a study from Parton et al. showed that α-melanocyte-stimulating hormone (α-MSH) release on hypothalamic chunks is increased in response to increased glucose level (Parton et al., 2007). This shows that POMC neurons can be activated by increased glucose level. Nevertheless, to our knowledge, no study has shown that glucose directly modulates the activity of POMC neurons. One could hypothesize that the modulation of POMC activity by glucose is due to presynaptic inputs rather than a direct detection by

POMC neurons themselves. The hypothesis that POMC neurons would not detect changes in glucose level directly is supported by a recent work showing that the frequency of excitatory postsynaptic currents onto POMC neurons is modulated by glucose (Hu et al., 2014). To get further insight into the direct glucose-sensing properties of POMC neurons, we studied their activity using calcium-imaging on freshly dissociated hypothalamic cells from POMC-GFP mice (for detailed method, see Chretien et al., 2017). In such preparation, dissociated cells are not in contact from each other (**Figure 2**). Thus, this strategy allows the study of direct glucose-sensing properties of hypothalamic neurons (Dunn-Meynell et al., 2002; Kohno et al., 2003; Kang et al., 2004; Vazirani et al., 2013; Chretien et al., 2017). As presented in **Figure 2**, we found that ∼8% hypothalamic neurons tested were identified as HGE neurons as they harbor a transient increase in $[Ca^{2+}]_i$ in response to a raise in glucose level from 2.5 to 10 mM. Nevertheless, none of the HGE neurons identified were GFP-expressing neurons ($n = 11$; **Figure 2**). Altogether, these data plus those from the literature suggest that glucose-sensing neurons modulate the melanocortin system but that POMC neurons themselves do not directly detect changes in glucose level. Further studies are still needed to determine the identity of ARC GE and HGE neurons. Knowing better the identity of hypothalamic (and extra-hypothalamic) glucose-sensing neurons is necessary to decipher the physiological functions controlled by these neurons.

MOLECULAR MECHANISMS INVOLVED IN HYPOTHALAMIC GLUCOSE-SENSING NEURONS

The determination of the signaling pathways involved in the detection of changes in glucose level has aroused the scientific community for decades. Detailed mechanisms involved in GE and GI neurons can be found in the review from Routh et al. in this special issue. We will concentrate here on ionic channels and signaling pathways taking part in hypothalamic detection of increased glucose level. We will not discuss the role of the couple GLUT2/Glucokinase in the detection of increased glucose since it has been suggested a while ago. Interestingly, the most recent studies have rather shown their putative role in the detection of decreased glucose level and in the regulation of the counter-regulatory response to hypoglycemia (Dunn-Meynell et al., 2002; Marty et al., 2005; Kang et al., 2006).

Which Ion Channel(s) Are Involved in Glucose Detection?

To determine mechanisms of brain glucose-sensing, studies have initially tried to determine similarities and differences from the known gold-standard glucose-sensor, the pancreatic β-cell (Yang et al., 1999). Thus, Michael Ashford was the first to show that ATP-dependent potassium (K_{ATP}) channels are involved in glucose-sensing of GE neurons (Ashford et al., 1990a,b). Nevertheless, it is not possible to determine whether GE and/or HGE-like neurons were studied as those pioneer studies used large glucose changes from 0 to 10 mM. Later, studies from Vanessa Routh's laboratory demonstrated that K_{ATP} channels are indeed involved in the detection of decreased glucose level by GE neurons (2.5–0.1 mM level window; Song et al., 2001; Wang et al., 2004). Nevertheless, they showed that the change in K_{ATP} conductance plateau above 2.5 mM (Wang et al., 2004), suggesting that K_{ATP} channels could only mediate detection below this level, and consequently, only in GE neurons. In addition, Guy Rutter's group suggested that K_{ATP} channels would not be involved in glucose detection above 2.5 mM in a study showing that intracellular ATP level is not increased in hypothalamic neurons in response to increased glucose level from 3 to 15 mM (Ainscow et al., 2002). We confirmed that K_{ATP} channels are not involved in HGE neurons. Indeed, we found that these channels are essentially closed at 5 mM. We also showed that HGE neurons can be found in K_{ATP}-deficient mice (Fioramonti et al., 2004) whereas GE neurons cannot be detected in these mice (Miki et al., 2001). Yang et al previously suggested that K_{ATP}-independent mechanisms would be involved in 5–20 mM glucose detection as the K_{ATP}-opener diazoxide failed to prevent increased glucose detection (Yang et al., 1999). Instead, we found that a non-selective cationic conductance was involved in the glucose response of HGE neurons (Fioramonti et al., 2004). More recently, we demonstrated that transient-receptor-potential canonical type 3 (TRPC3) channels are required for glucose detection by HGE neurons (Chretien et al., 2017). Involvement of non-selective cationic conductance has also been identified in the response to glucose of GnRH neurons even though the identity of the channel responsible for glucose-sensing in these neurons is yet to be determined (Roland and Moenter, 2011b). TRPC channels are involved in the response to insulin and leptin of ARC kisspeptin and POMC neurons (Qiu et al., 2010, 2011, 2014, 2017). Together, these studies open new line of research for hypothalamic glucose- and, more generally, nutrient-sensing mechanisms (Chretien et al., 2017).

Role of the Mitochondrial Reactive Oxygen Species (mROS) in Glucose Detection

The mitochondrial respiratory chain represents one the main sources of ROS. Increasing reduced equivalents ($NADH,H^+$ and $FADH_2$) as a result of glucose oxidation leads to increased electron transfer chain activity, generating an elevated but physiological superoxide anions production. Our group showed that cerebral injection of glucose in rodents leads to a short-lived mROS signaling, in the form of H_2O_2 (see for review Leloup et al., 2011). We and others also showed that this increase in mROS level is necessary for the of glucose-sensing neurons to increased glucose (Chretien et al., 2017) and the regulation of food intake and insulin secretion (Leloup et al., 2006; Andrews et al., 2008; Colombani et al., 2009; Carneiro et al., 2012). Thus, increased mROS levels, rather than just the ATP/ADP ratio, constitute a signal that mediates the stimulatory effect of glucose on some hypothalamic neurons. Therefore, mROS signaling is consistent with the NADH mechanism suggested earlier for the glucose sensing mechanism (Yang et al., 1999; Ainscow and Rutter, 2002). In addition, the fact that TRPC3 channels form a redox-sensitive

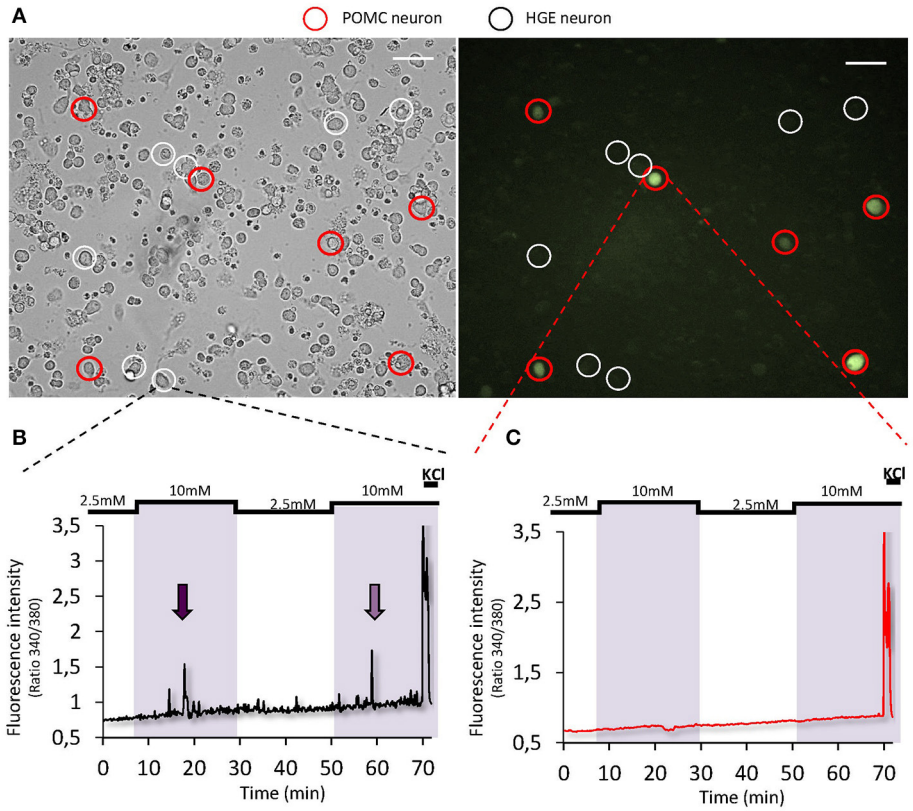


FIGURE 2 | Glucose-sensing properties of POMC neurons. **(A)** Representative bright-field (left panel) or fluorescence (right panel) images of cultured dissociated MBH neurons from POMC-GFP mice (×20 objective, scale bar = 40 μm; Fioramonti et al., 2007). Detailed methods for cell culture preparation and calcium imaging recording is explained in Chretien et al. (2017). **(B,C)** Representative calcium imaging traces of a HGE **(B)** or a non-glucose-sensing POMC neuron **(C)** in response to 2 consecutives increased glucose level from 2.5 to 10 mM.

complex reinforces the role of mROS in the signaling involved in glucose detection.

Mitochondria are organized into a tubular network that continuously changes its shape and motility, mediated by fission and fusion mechanisms. Mitochondrial dynamics (fusion and fission) have been linked to the balance between energy demand and nutrient supply. Our group suggested that mitochondria dynamics (fission) play a significant role in mROS production in response to increased glucose level. In the MBH, we highlighted glucose-induced Drp1-dependent mitochondrial fission is an upstream regulator for mROS signaling, and consequently, a key mechanism in hypothalamic glucose sensing (Carneiro et al., 2012). More recently, Diano's and Claret's teams showed that fission and fusion mechanisms are involved in the detection of increased or decreased glucose level by hypothalamic POMC or SF1 neurons (Schneeberger et al., 2013; Toda et al., 2016; Ramirez et al., 2017; Santoro et al., 2017). Even though the involvement of mitochondria dynamics seems to be a critical mechanism involved in glucose detection and in the regulation of energy and glucose homeostasis, the signaling pathways leading to such changes in mitochondria morphology is unclear and needs to be studied further. The idea that such mechanisms are specific to glucose-sensing neurons also needs to be addressed.

What about *Metabolism-Independent* Glucose-Sensing Mechanisms?

Some studies highlighted that *metabolism-independent* mechanisms take part in neuronal glucose-sensing. Thus, it has been shown that the sodium-glucose cotransporters (SGLT)-inhibitor phloridzin, blocks the response to increased glucose level of some hypothalamic neurons (Yang et al., 1999; O'Malley et al., 2006). Furthermore, the non-metabolizable substrate of SGLTs, α-methylglucopyranoside, mimics the effect of increased glucose level on a majority of HGE-like neurons (O'Malley et al., 2006). SGLT1 or SGLT3 could be the transporters responsible for glucose detection as they are expressed in hypothalamic neurons. The hypothesis on SGLT3 is particularly interesting since this transporter is expressed in human cholinergic neurons where it acts as a glucose-activated sodium channel which does not transport glucose (Diez-Sampedro et al., 2003). Interestingly, SGLT3 has been shown to be the sensor mediating glucose detection of the portal vein (Delaere et al., 2012).

Another *metabolism-independent* mechanism has been suggested to mediate glucose detection in the hypothalamus through sweet taste receptors. These receptors present in papillae of the tongue mediate the sweet sensation of sugars

and sugar substitute (Laffitte et al., 2014). These receptors are heterodimers composed of T1R2/T1R3 subunits which have also been identified in several extra-gustatory tissues and cells including the pancreatic β-cell and the brain (Laffitte et al., 2014). In the brain, the expression of T1R2 and T1R3 subunits is particularly enriched in the ARC (Ren et al., 2009; Herrera-Moro Chao et al., 2016). Recently, Yada's group showed that half of the HGE-like neurons in the ARC (activated by the increase in glucose level from 1 to 10 mM) are excited by the application of the sweetener sucralose. They also showed that the sweet taste receptor inhibitor gurmarin, blocks the response to glucose of 2/3 of HGE-like neurons (Kohno et al., 2016). Finally, in this study, they also show that sucralose activates neurons of the ARC which are not POMC neurons, reinforcing the idea that POMC neurons do not detect directly changes in glucose level. These data highlight a new mechanism involved in hypothalamic glucose-sensing. Further studies are however necessary to determine the physiological role of the sweet taste receptors expressed in hypothalamic glucose-sensing neurons in the control of energy homeostasis. It will also be interesting to determine the ionic channel mediating the effect of sweeteners or glucose downstream sweet taste receptor. The involvement of K_{ATP} or TRPC channels has always been linked to *metabolic-dependent* mechanisms. However, one could hypothesize that these channels mediate sweet taste receptor-dependent glucose-sensing. Twik-related acid-sensitive potassium-like (TASK) 1/3 channels could also be a relevant candidate as they take part in *metabolism-independent* mechanism of orexin GI neurons in the LH (Gonzalez et al., 2008, 2009).

CONCLUSION

We described briefly the recent advances made in the comprehension of mechanisms involved in brain detection of increased glucose level. We highlighted the role of TRPC channels, ROS, mitochondria dynamics and pointed out that POMC neurons might not be directly sensing glucose. Nevertheless, additional studies are still needed to determine parts of the puzzle linking the pathways and whether they are activated in a same glucose-sensing neuron or whether several mechanisms are recruited in distinct neurons. We also highlighted that new lines of research are still needed to further characterize the nature of glucose-sensing neurons and to determine potential new locations within the brain. These data will help better understanding of the various physiological functions they control.

ETHICS STATEMENT

All procedures were performed in agreement with European Directive 2010/63/UE and approved by the French Ministry of Research and the local ethics committee of the University of Burgundy (C2EA Grand Campus Dijon N°105; agreement N°02404.02).

AUTHOR CONTRIBUTIONS

Research data, CC. Wrote the manuscript, XF, LP, CC, and CL. Review/Edited the manuscript, XF, CC, LP, CL.

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Hypoglycemia: Role of Hypothalamic Glucose-Inhibited (GI) Neurons in Detection and Correction

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Hypoglycemia is a profound threat to the brain since glucose is its primary fuel. As a result, glucose sensors are widely located in the central nervous system and periphery. In this perspective we will focus on the role of hypothalamic glucose-inhibited (GI) neurons in sensing and correcting hypoglycemia. In particular, we will discuss GI neurons in the ventromedial hypothalamus (VMH) which express neuronal nitric oxide synthase (nNOS) and in the perifornical hypothalamus (PFH) which express orexin. The ability of VMH nNOS-GI neurons to depolarize in low glucose closely parallels the hormonal response to hypoglycemia which stimulates gluconeogenesis. We have found that nitric oxide (NO) production in low glucose is dependent on oxidative status. In this perspective we will discuss the potential relevance of our work showing that enhancing the glutathione antioxidant system prevents hypoglycemia associated autonomic failure (HAAF) in non-diabetic rats whereas VMH overexpression of the thioredoxin antioxidant system restores hypoglycemia counterregulation in rats with type 1 diabetes.We will also address the potential role of the orexin-GI neurons in the arousal response needed for hypoglycemia awareness which leads to behavioral correction (e.g., food intake, glucose administration). The potential relationship between the hypothalamic sensors and the neurocircuitry in the hindbrain and portal mesenteric vein which is critical for hypoglycemia correction will then be discussed.

Keywords: ventromedial hypothalamus, neuronal nitric oxide synthase, perifornical hypothalamus, orexin, oxidative stress, hypoglycemia associated autonomic failure, hypoglycemia unawareness

INTRODUCTION

Glucose is the primary fuel of the brain. For this reason, neural circuits exist which sense declining glucose levels and restore euglycemia. These circuits evolved to protect against falls in blood glucose during exercise or fasting. In the modern world, they are also the critical defense against life-threatening insulin-induced hypoglycemia (Cryer, 1981). When glucose levels fall below 80 mg/dl a sequential hormonal response is initiated to restore euglycemia. Insulin secretion ceases followed by the stepwise secretion of the gluconeogenic hormones glucagon, epinephrine, cortisone and growth hormone as glucose levels continue to fall toward ∼60 mg/dl (Amiel et al., 1988; Mitrakou et al., 1991). This hormonal response is referred to as the counterregulatory response to hypoglycemia

(CRR). Decreasing blood glucose levels below 60 mg/dl also initiates neurogenic (e.g., palpitations, sweating) followed by neuroglycopenic (tired/drowsy, difficulty thinking) symptoms near 50 mg/dl which together comprise hypoglycemia awareness (Mitrakou et al., 1991). Hypoglycemia awareness promotes behavioral responses such as feeding or glucose administration (Cryer, 1981; Amiel et al., 1988).

Recurrent iatrogenic hypoglycemia (RH) is the chief barrier to maintaining optimal glycemic control using intensive insulin therapy in type 1 and advanced type 2 diabetes mellitus. RH leads to the deleterious syndromes known as hypoglycemia associated autonomic failure (HAAF) and hypoglycemia unawareness. During HAAF, the glycemic threshold for detection of hypoglycemia shifts to lower glucose levels. When this occurs CRR initiation can be dangerously delayed and blunted in magnitude. Similarly during hypoglycemia unawareness neurogenic and neuroglycopenic symptoms do not warn an individual of impending hypoglycemia and thus prevent behavioral correction (Amiel et al., 1988; Mitrakou et al., 1991; Cryer, 2013). Hypoglycemia unawareness was thought to result from failure of autonomic activation and potentially autonomic neuropathy (Towler et al., 1993). However, more recent studies suggest that heightened arousal is necessary to recognize and interpret the peripheral neurogenic symptoms. This led to the hypothesis that incorrect interpretation of peripheral symptoms of hypoglycemia is a key component of hypoglycemia unawareness (Otlivanchik et al., 2015, 2016).

Robust initiation of corrective mechanisms is particularly important for diabetic patients since even without clinical HAAF glucagon release in response to hypoglycemia is nearly absent in type 1 and advanced type 2 diabetic patients (Bottini et al., 1997; Segel et al., 2002; Cryer, 2005; Israelian et al., 2006; Siafarikas et al., 2012; Oyer, 2013). Thus, improving and preserving proper hypoglycemia detection is critical for diabetes management. Animal studies have described glucose sensors throughout the brain and in many peripheral tissues (Routh et al., 2012). However, those within the hypothalamus have been the most extensively studied, especially in relation to HAAF and hypoglycemia unawareness (Routh, 2003; Song and Routh, 2006; Kang et al., 2008; Fioramonti et al., 2010; Chan et al., 2013; Fan et al., 2015; Otlivanchik et al., 2015, 2016). In this article we will focus on hypothalamic sensors and their involvement in these pathologies resulting from RH. We will then explore their potential interaction with the important neural circuits of the hindbrain and portal vein of the liver which are also key for the CRR (Ritter et al., 2011; Donovan and Watts, 2014).

HYPOTHALAMIC GLUCOSE SENSING AND HYPOGLYCEMIA DETECTION

Strong evidence in rodents supports a role for ventromedial hypothalamus (VMH) glucose sensors in the hormonal CRR (Borg et al., 1994, 1995, 1997; Tong et al., 2007) as well as in glucoprivic feeding (Dunn-Meynell et al., 2009). The VMH possesses two major subtypes of glucose-sensing neurons (Anand et al., 1964; Oomura et al., 1964). Glucose-excited (GE) neurons increase while glucose-inhibited (GI) neurons decrease their activity as glucose levels rise (Ashford et al., 1990; Song et al., 2001). As mentioned above the ability to sense glucose has been observed throughout the brain in neurons as well as in glial cells (Levin, 2000; Marty et al., 2005; Zhou et al., 2010; Frayling et al., 2011; Melnick et al., 2011; Routh et al., 2012). It is true that any glucose sensing cell within the CNS may potentially contribute to the regulation of the CRR. However, the glucose sensitivity of VMH glucose sensing neurons, especially that of GI neurons, parallels the ability of the brain to sense and respond to hypoglycemia (McCrimmon et al., 2006; Song and Routh, 2006; Kang et al., 2008; Diggs-Andrews et al., 2010; Fioramonti et al., 2010, 2013). This has important implications for preserving the CRR during HAAF and diabetes. Thus, this perspective focuses primarily on these neurons. However, this does not preclude important roles for other central glucose sensors in regulating the CRR.

VMH Glucose Sensing Neurons

Like the pancreatic β-cell, glucose sensing by VMH GE neurons depends on a low-affinity hexokinase IV isoform, glucokinase (GK) and the ATP-sensitive K^+ channel (K_{ATP})(Ashford et al., 1990; Dunn-Meynell et al., 2002; Kang et al., 2006). GK also mediates glucose sensing in VMH GI neurons (Dunn-Meynell et al., 2002; Kang et al., 2006). However, in GI neurons, decreased glucose activates the cellular fuel sensor AMP activated protein kinase (AMPK). AMPK phosphorylates the neuronal nitric oxide (NO) synthase (nNOS) leading to NO production. NO then binds to its receptor, soluble guanylyl cyclase (sGC), which increases cyclic guanosine monophosphate (cGMP) production. Increased cGMP causes a further activation of AMPK. This cGMP mediated activation of AMPK leads to closure of a chloride channel, possibly the cystic fibrosis transmembrane regulator (CFTR), depolarization, and increased neuronal activity (Murphy et al., 2009a). These studies demonstrate that NO production is required for depolarization of GI neurons in low glucose. However, NO is a diffusible gas which acts as a retrograde signal at synapses and plays a role in plasticity (Edelmann et al., 2007; Dejanovic and Schwarz, 2014). NO diffusion also regulates neuroendocrine processes via recruitment of adjacent neurons (Bellefontaine et al., 2014). Whether NO diffusion within synapses or to adjacent cells plays a role in glucose sensing is not known.

The ability of VMH GI neurons to be activated by low glucose closely parallels the magnitude of the CRR. For example, as seen for CRR initiation, glucose levels must fall further after RH before GI neurons are activated (Song and Routh, 2006). AMPK, nNOS and sGC inhibition in the perfusion solution *in vitro* and in the VMH *in vivo*, respectively, blocks activation of GI neurons in low glucose and impairs the CRR (McCrimmon et al., 2008; Murphy et al., 2009a; Fioramonti et al., 2010). Hypo- or hyperglycemia induced-oxidative/nitrosative stress may underlie both the blunted response of VMH GI neurons to decreased glucose and CRR impairment (Colombani et al.,

2009; Fioramonti et al., 2013). Oxidative and nitrosative stress cause s-nitrosation of sGC (and other proteins)(Jaffrey et al., 2001). Nitrosated sGC is resistant to NO (Sayed et al., 2007). Insulin-induced hypoglycemia increases sGC s-nitrosation and VMH injection with a nitrosating agent impairs the CRR. Furthermore, reducing oxidative stress with the glutathione precursor, N-acetylcysteine, in non-diabetic rats completely prevented altered glucose sensing by VMH GI neurons and CRR impairment during HAAF (Fioramonti et al., 2013). Diabetic hyperglycemia also induces oxidative stress and reduces both activation of VMH GI neurons by decreased glucose and glucagon secretion during hypoglycemia (Cryer et al., 2003; Canabal et al., 2007a; Cardoso et al., 2010; Zhou and Routh, 2017). However, N-acetylcysteine did not normalize glucose sensing and CRR initiation in diabetic animals (Zhou and Routh, 2017). Diabetic hypoglycemia is associated with impairment of the VMH thioredoxin antioxidant system via activation of the inhibitory enzyme, thioredoxin interacting protein or TXNIP (Blouet and Schwartz, 2011). VMH thioredoxin overexpression completely normalized GI neuronal glucose sensing, glycemia recovery, and glucagon secretion in rats with type 1 diabetes mellitus. However, VMH thioredoxin overexpression did not prevent HAAF in these animals (Zhou and Routh, 2017). These findings are interesting for two reasons. First, impaired glucagon secretion in diabetes is often thought to be due to local factors at the level of the alpha cell. However, these studies suggest a direct effect of the VMH on pancreatic alpha cell function during the CRR. Second, the effects of N-acetylcysteine were more pronounced during RH whereas VMH thioredoxin overexpression was more effective in diabetes. Neither antioxidant system alone compensated for HAAF when it occurred during diabetes. This suggests that a combination of antioxidants might be necessary to preserve the CRR when HAAF occurs during diabetes. **Figure 1** illustrated the respective roles of the VMH glutathione and thioredoxin systems in HAAF and diabetes.

VMH thioredoxin overexpression had 2 additional interesting and unexpected effects. First, while virtually all animals injected with the control vector developed diabetes after one injection of the β-cell toxin streptozotocin, one quarter of the VMH thioredoxin overexpressing animals did not (Zhou and Routh, 2017). This suggests that the VMH has a protective role on the pancreatic β-cell, perhaps by reducing inflammation. However, it should be noted that a second injection of toxin led to diabetes in the resistant animals. Nevertheless, this putative protective effect of enhanced VMH antioxidant defense on the pancreas deserves further study. The second unexpected effect of VMH thioredoxin overexpression was a reduction in the amount of insulin required to manage blood glucose levels (Zhou and Routh, 2017). Whether this is due to reduced basal hepatic glucose output, increased glucose uptake by insulin sensitive tissues, or other changes in metabolism is not known. However, this finding is consistent with a role of the VMH in peripheral insulin sensitivity and glucose homeostasis (Steffens et al., 1988; Martins et al., 2016; Coutinho et al., 2017). Moreover a role for VMH oxidative status in glucose homeostasis is consistent with a recent report that uncoupling protein regulates VMH glucose sensing and peripheral glycemia by decreasing oxidative stress (Toda et al., 2016).

Further support for a role of VMH GI-nNOS neurons in the CRR derives from their relationship with glutamate signaling. The VMH possesses high expression of mRNA encoding the vesicular glutamate transporter VGLUT2, a marker for glutamate neurons (Ziegler et al., 2002). Virtually all VMH nNOS neurons are glutamatergic (Chachlaki et al., 2017). Since we find that 95% of VMH GI neurons produce NO in decreased glucose and GI neurons are absent in mice lacking nNOS, it is very likely that VMH GI neurons are glutamatergic (Canabal et al., 2007b; Fioramonti et al., 2010). Mice lacking VGLUT2 selectively in steroidogenic factor 1 neurons that mark the VMH have defective CRR to insulin-induced hypoglycemia (Tong et al., 2007). Taken together, these data lead us to hypothesize that maintaining normal oxidative balance in VMH glutamatergic nNOS expressing GI neurons is necessary for the CRR and potentially other aspects of glucose homeostasis.

Orexin Glucose Sensing Neurons and Hypoglycemia Unawareness

Hypothalamic orexin neurons are also GI neurons (Burdakov et al., 2005). Perifornical hypothalamus (PFH) orexin neurons play a role in epinephrine secretion during hypoglycemia (Otlivanchik et al., 2015; Korim et al., 2016). Orexin neurons may also contribute to hypoglycemia awareness. Otlivanchik et al showed that one episode of insulin-hypoglycemia in the conditioned side of a conditioned place preference (CPP) box reversed the preference for that side of the box on the subsequent day. They interpreted this finding to mean that the animal was aware of hypoglycemia, associated it with the formerly preferred side of the box and developed an aversion to that side. Interestingly, 3 consecutive daily episodes of hypoglycemia *in the home cage* prevented subsequent hypoglycemia on the preferred side from disrupting the CPP. This suggests that the animal exhibited hypoglycemia unawareness and thus did not develop an aversion. Interestingly, systemic injection of a brain permeant orexin antagonist mimicked hypoglycemia unawareness (Otlivanchik et al., 2016). PFH orexin neurons facilitate arousal via their projections to the tuberomammillary nucleus histamine neurons (Sundvik and Panula, 2015). Thus, PFH orexin-GI neurons may play a role in hypoglycemia awareness and be a target for treating hypoglycemia unawareness.

An interesting characteristic of the glucose sensitivity of VMH and orexin GI neurons is regulation by metabolic state. For example, fasting increases, while the satiety hormone leptin decreases the activation of VMH and orexin GI neurons, as well as that of arcuate nucleus neuropeptide Y expressing GI neurons, by decreased glucose (Murphy et al., 2009b; Sheng et al., 2014). The hunger hormone ghrelin increases the activation of orexin-GI neurons in low glucose (Sheng et al., 2014). Thus, during energy deficit when low glucose is a greater threat hypoglycemia may produce a stronger activation of hypothalamic GI neurons.

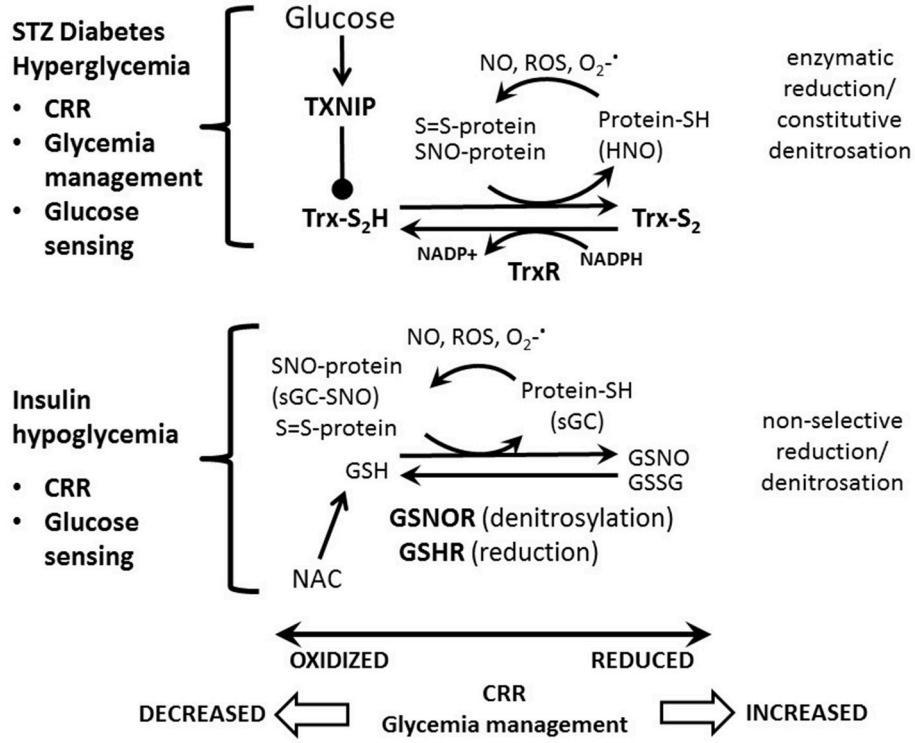

FIGURE 1 | Model for the respective roles of the VMH glutathione and thioredoxin systems in HAAF and diabetes. Increased nitric oxide (NO), reactive oxygen species (ROS) and/or superoxide ($O_2^-\cdot$) cause protein S-nitrosation (SNO-protein) and oxidation (S = S-protein). We have shown that soluble guanylyl cyclase is S-nitrosated (sGC-SNO) during hypoglycemia leading to impaired glucose sensing and counterregulatory response (CRR). Reduced glutathione (GSH) causes a non-selective reduction and/or denitrosation of oxidized proteins (protein-SH; sGC) becoming oxidized (GSSG) or nitrosated (GSNO) itself in the process. GSH is regenerated by the enzymes glutathione reductase (GSHR) and nitrosated glutathione reductase (GSNOR) (Nordberg and Arnér, 2001). Oral administration of the glutathione precursor, N-acetylcysteine (NAC), completely prevents hypoglycemia associated autonomic failure (HAAF) and preserves glucose sensing by VMH GI neurons in non-diabetic but not diabetic animals. Reduced thioredoxin (Trx-S_2H) enzymatically catalyzes protein reduction and denitrosation becoming oxidized in the process (Trx-S_2). During denitrosation HNO is released. Trx-S_2H is regenerated by the enzyme thioredoxin reductase (TrxR) in an NADPH dependent fashion. The glucose activated enzyme, thioredoxin interacting protein (TXNIP) reduces Trx-S_2H activity (Stoyanovsky et al., 2005). VMH thioredoxin overexpression completely normalizes glucose sensing by GI neurons and the CRR in diabetes alone but not when HAAF occurs during diabetes. VMH thioredoxin overexpression also reduces the insulin required to manage hyperglycemia in diabetes. Enzyme abbreviations are bold-faced.

This would enable a more robust response to hypoglycemia despite diminished energy reserves.

RELATIONSHIP BETWEEN HYPOTHALAMIC, HINDBRAIN AND PORTAL-MESENTERIC VEIN (PMV) GLUCOSE SENSORS

Glucose sensors in the hindbrain and PMV are critical for the CRR as detailed in several comprehensive review articles (Ritter et al., 2011; Routh et al., 2012; Donovan and Watts, 2014). Ritter and colleagues show that specific clusters of catecholamine neurons within the C1 cell groups (C1r, C1m, A1/C1) of the rostral ventral lateral medulla (RVLM) in rodents are essential for individual components of hypoglycemia correction including epinephrine and corticosterone secretion as well as glucoprivic feeding (Ritter et al., 1981, 1998, 2001, 2006; Li et al., 2017). The feeding and corticosterone response is mediated by forebrain projections to hypothalamus (i.e. paraventricular nucleus, PFH) whereas the adrenomedullary response is mediated by bulbospinal projections (Ritter et al., 2001, 2006; Li et al., 2015b). Recent work by this group has shown that RVLM catecholamine neurons reciprocally innervate PFH orexin neurons in order to control glucoprivic feeding responses (Li et al., 2015a,b). These authors present the intriguing hypothesis that this interaction may enable the orexin system to coordinate arousal with feeding behavior.

Glucose sensors within the PMV are also essential for the hormonal CRR in experimental models (Donovan et al., 1994; Hevener et al., 2000; Fujita et al., 2007; Donovan and Watts, 2014). Interestingly, the role of the PMV glucose sensor in the CRR is dependent on the rate of glucose decline. That is, PMV glucose sensors dominate during slow-onset hypoglycemia "($\leq$1 mg/dL • min^{-1})." In contrast, CNS sensors dominate when glucose levels fall quickly ($\geq$2 mg · dl^{-1} · min^{-1}) (Matveyenko et al., 2007; Saberi et al., 2008; Bohland et al., 2014). The former corresponds to a drop in

blood glucose from euglycemia (∼100 mg/dl) to hypoglycemia (60 mg/dl) within approximately 60 min. while in the latter blood glucose would decrease to 60 mg/dl within 20 min. While slower rates of decline predominate during insulin therapy in humans, rapid decline occurs at an incidence of ∼30% (Kovatchev et al., 2005). An additional issue to be considered when interpreting these data is that the studies of the relative role of PMV and CNS glucose sensors on the CRR were done in non-diabetic controls. Thus, whether the starting glycemia plays a role in CNS vs. peripheral detection is not known. Interestingly, hyperglycemia *per se* significantly reduces activation of VMH GI neurons in low glucose (Canabal et al., 2007a).

The cellular mechanism of PMV glucose sensing is still unknown. However, the effect of PMV glucose sensors on the sympathoadrenal response during slow-onset hypoglycemia is dependent on sympathetic afferents to the hindbrain (Bohland et al., 2014). Moreover, hindbrain catecholamine projections to the hypothalamus are essential for sympathoadrenal activation and glucagon secretion during slow- but not fast-onset hypoglycemia (Jokiaho et al., 2014). Together, these data suggest that an interaction between glucose sensors in the PMV and the hypothalamically projecting hindbrain catecholamine neurons is required for the secretion of epinephrine and glucagon when glucose levels fall slowly. In contrast, the hypothalamic glucose sensors dominate when glucose levels fall quickly. It is also possible that any or all of these sensors play a role in the inhibition of insulin secretion during hypoglycemia.

PERSPECTIVE

Clearly, hypoglycemia is a severe threat to the brain and therefore multiple neurocircuits have evolved to sense and restore euglycemia. The data discussed in this review suggest hierarchical control of the CRR and feeding responses to hypoglycemia. A model for such a hierarchical organization can be found in an excellent recent review (Donovan and Watts, 2014). These data also suggest some degree of specificity in control of different aspects of the response to hypoglycemia by the different glucose sensing regions. That is, slow-onset hypoglycemia is primarily sensed by the PMV and information is sequentially relayed to the hindbrain catecholamine neurons and hypothalamus in order to increase glucagon and epinephrine secretion. It is likely that this information undergoes integration and further processing at the level of the hindbrain, given the response of the catecholamine neurons to glucoprivic agents. In addition activation of these hindbrain glucose sensors initiates feeding and corticosterone responses through hypothalamic projections. On the other hand when glucose levels fall rapidly, the threat to the brain is more severe and hypothalamic glucose sensors are fully capable of restoring euglycemia.

Based on our data we hypothesize that even under slow-onset hypoglycemia the VMH glucose sensors integrate the information and potentially enhance the CRR during energy deficit. That is, VMH nNOS (glutamatergic?) GI neurons may be important in setting the gain of the response to compensate for the current energetic status of the body. In particular, the degree of activation of VMH nNOS GI neurons in low glucose is closely coupled to the magnitude of the hormonal CRR. These GI neurons are particularly sensitive to oxidative stress and enhancing antioxidant defenses during either RH or diabetes is sufficient to preserve the CRR. Further work needs to be done in order to determine whether this strategy will be effective when HAAF occurs during diabetes. Interestingly, enhancing VMH antioxidant defenses appears to be very important for restoring glucagon responses during early diabetes as well as aiding in glycemic management. In the future it would be interesting to determine whether GI neurons are playing a role in both of these VMH functions. It will also be important to determine whether the effects of thioredoxin persist in type 2 diabetes mellitus or in other models of type 1 diabetes. Finally, the PFH orexin-GI neurons may play a role in hypoglycemia awareness by coupling arousal and behavioral responses during hypoglycemia.

AUTHOR CONTRIBUTIONS

CZ, ST, BK, and CG wrote portions of the first draft with CZ making the greatest contribution. VR revised the first draft critically for important intellectual content, wrote the Perspectives section and generated the final draft of the manuscript.

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Nesfatin-1 in the Lateral Parabrachial Nucleus Inhibits Food Intake, Modulates Excitability of Glucosensing Neurons, and Enhances UCP1 Expression in Brown Adipose Tissue

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Nesfatin-1, an 82-amino acid neuropeptide, has been shown to induce anorexia and energy expenditure. Food intake is decreased in *ad libitum*-fed rats following injections of nesfatin-1 into the lateral, third, or fourth ventricles of the brain. Although the lateral parabrachial nucleus (LPBN) is a key regulator of feeding behavior and thermogenesis, the role of nesfatin-1 in this structure has not yet been delineated. We found that intra-LPBN microinjections of nesfatin-1 significantly reduced nocturnal cumulative food intake and average meal sizes without affecting meal numbers in rats. Because glucose sensitive neurons are involved in glucoprivic feeding and glucose homeostasis, we examined the effect of nesfatin-1 on the excitability of LPBN glucosensing neurons. *In vivo* electrophysiological recordings from LPBN glucose sensitive neurons showed that nesfatin-1 (1.5×10^{-8} M) excited most of the glucose-inhibited neurons. Chronic administration of nesfatin-1 into the LPBN of rats reduced body weight gain and enhanced the expression of uncoupling protein 1 (UCP1) in brown adipose tissue (BAT) over a 10-day period. Furthermore, the effects of nesfatin-1 on food intake, body weight, and BAT were attenuated by treatment with the melanocortin antagonist SHU9119. These results demonstrate that nesfatin-1 in LPBN inhibited food intake, modulated excitability of glucosensing neurons and enhanced UCP1 expression in BAT via the melanocortin system.

Keywords: food intake, energy expenditure, glucose sensitive neurons, lateral parabrachial nucleus, nesfatin-1, SHU9119, rat

Abbreviations: LPBN, lateral parabrachial nucleus; NUCB2, nucleobindin-2; PVN, paraventricular nucleus; GLP-1, glucagon-like peptide-1; UCP1, uncoupling protein 1; BAT, brown adipose tissue; WAT, white adipose tissue; GE, glucose excitatory; GI, glucose inhibitory.

INTRODUCTION

Nesfatin-1 was identified as a satiety molecule by Oh et al. (2006), and its precursor, nucleobindin-2 (NUCB2), is associated with severe obesity in humans (Zegers et al., 2012). NUCB2/nesfatin-1 is widely expressed in the periphery (García et al., 2012) and in the central nervous system. It is expressed mainly in the paraventricular nucleus of hypothalamus (PVN), arcuate nucleus, lateral hypothalamic area, solitary tract nucleus, and the spinal cord (Oh et al., 2006; Brailoiu et al., 2007; Maejima et al., 2009). Additionally, expression of NUCB2/nesfatin-1 has been detected in the lateral parabrachial nucleus (LPBN) (Goebel et al., 2009, 2011a).

The LPBN is involved in appetite regulation (Wu et al., 2012; Garfield et al., 2015; Roman et al., 2016), thermostasis (Morrison et al., 2012), glucose homeostasis (Flak et al., 2014), and malaise (Carter et al., 2015). Various feeding-related peptides, including leptin (Alhadeff et al., 2014a), glucagon-like peptide-1 (GLP-1) (Alhadeff et al., 2014b; Richard et al., 2014), amylin (Lutz, 2010), and cholecystokinin (Becskei et al., 2007), act directly on the LPBN to reduce food intake. However, evidence for the role of nesfatin-1 in the LPBN has hitherto been lacking. Acute injections of nesfatin-1 into the lateral (Maejima et al., 2009), third (Oh et al., 2006; Watts and Donovan, 2010), or fourth (Yosten and Samson, 2009) ventricles of the brain decrease food intake in rats and mice. The anorexigenic mechanisms of nesfatin-1 encompass several hypothalamic and medullary pathways in a leptin-independent manner (Oh et al., 2006; Maejima et al., 2009; Stengel et al., 2009a). The crosstalk between nesfatin-1 and corticotropin-releasing factor, oxytocin, and melanocortin pathways has yet to be clarified.

Glucose sensitive neurons are involved in glucoprivic feeding and glucose homeostasis. Recently they have received more attention due to their potential role in regulating appetite (Routh, 2002; Watts and Donovan, 2010). Previously, we discovered that nesfatin-1 inhibits food intake by influencing the firing rates of glucose sensitive neurons in the hypothalamus (Chen et al., 2012) and the dorsal vagal complex (Dong et al., 2014). It was also shown recently that the glucose-sensitive territory in the brain includes the LPBN in addition to the hypothalamic and hindbrain centers (de Araujo, 2014; Garfield et al., 2014). Moreover, GLP-1 decreases food intake and increases the firing rates of LPBN neurons (Richard et al., 2014). However, the types of neurons responding to GLP-1 have not been identified. Here, we investigate the influence of nesfatin-1 in the LPBN on the firing rate of glucosensing neurons in this area and its effect on feeding behaviors.

Central administration of nesfatin-1 has been shown to increase energy expenditure in rodents (Konczol et al., 2012; Wernecke et al., 2014). In addition, its anorexigenic effects are abolished by a melanocortin 3/4 receptor antagonist, SHU9119 (Oh et al., 2006). In fact, the central melanocortin system and its interactions with other neuroendocrine pathways have emerged as key regulators of appetite and thermogenesis (Myers and Olson, 2012; Krashes et al., 2016). A mutation in the melanocortin-4 receptor has been associated with severe obesity and inducing lower energy expenditure in humans (Ma et al., 2004). Administration of the melanocortin 3/4 receptor agonist MTII into the LPBN of rodents significantly reduces nocturnal food intake and increases core temperature and heart rate without affecting spontaneous activity (Skibicka and Grill, 2009). Intriguingly, Williams et al. (2003) found that the forebrain and hindbrain were equally effective at stimulating MTII-induced expression of uncoupling protein 1 (UCP1), a primary effector of thermogenesis, in brown adipose tissue (BAT). We hypothesize that the LPBN melanocortin-signaling pathway is involved in the effects of nesfatin-1 on food intake, energy expenditure, and body weight. We tested this hypothesis by determining whether the effects of nesfatin-1 on energy balance are blocked by SHU9119.

These studies utilized a combination of behavioral, electrophysiological, and pharmacological techniques to test the hypothesis that nesfatin-1 in the LPBN is involved in energy metabolism.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (Qingdao Institute of Drug Control) weighing 270–300 g were housed in a temperature-controlled ($23 \pm 2^{\circ}C$) animal room (illumination from 7:00 to 19:00). Rats were group-housed (four per cage) and allowed free access to standard food and tap water for at least 1 week to adapt to their surroundings. The protocols were approved by the Qingdao University Animal Care and Use Committee in accordance with the National Institutes of Health guidelines.

Surgery

Rats were anesthetized with chloral hydrate (80 mg/ml, 0.5 ml/100 g of body weight, i.p.) and positioned in a stereotaxic apparatus (SN-3; Narishige, Tokyo, Japan) for implantation of a 26-gauge chronic guide cannula above the right lateral LPBN. Stereotaxic coordinates were obtained from the brain atlas of Paxinos and Watson (Gaxinos and Watson, 2007): 9.0 mm caudal to bregma, 2.2 mm right lateral, and 6.4 mm ventral to the skull (Supplementary Figures 1A,B). After quickly and accurately reaching the target depth, microabsorbent cotton was used for local hemostasis. The cannula was fixed in place with dental cement and a stainless-steel screw. A 28-gauge obturator (the same length as the cannula) was placed in the cannula.

After completion of all experiments, the rats were sacrificed and their brains were removed. Cannula placement was verified by injections of 0.5 μl pontamine sky blue, and the brains were fixed in a 4% formaldehyde solution and cut on a freezing microtome (Kryostat 1,720; Leica, Germany). Data from rats with injection sites in the correct location were included in the analyses (Supplementary Figure 2A).

Experimental Procedures

Experiment 1: Effects of Nesfatin-1 in the LPBN on Nocturnal Food Intake and Meal Patterns

After the surgery, rats ($n = 32$) were housed individually for recovery for 5–7 days before being placed in metabolic cages (Feeding and Activity Analyser 47552-002; Ugo Basile, Italy) and habituated to powdered standard chow (Qingdao

Daren Fortune Animal Technology) which was crashed by the crusher (Ronghao, RH-600A) and tap water (Ulman et al., 2008). The metabolic cages were monitored with load cells incorporating data acquisition software (51,800, Feed-Drink Monitoring System Ver. 1.31; Ugo Basile, Italy). Load cells sense the load of food and liquid every 5 s, thus recording their consumption, and monitor the frequency of food/liquid uptake by detecting stable or unstable weights. The crumbs produced by rats were collected in the front compartment for a precise evaluation of food consumption. Animals were administered drugs with a 28-gauge injector via a microsyringe extended 0.8 mm below the guide cannula. The drugs were delivered to the nuclei parenchyma at a speed of 0.25 μl/min. After the injection, the injector was kept in the cannula for another 5 min (Dong et al., 2014). Prior to testing, rats received saline injections (0.5 μl of 0.9% NaCl) through the guide cannula for 3 days as training for drug injections. On the experimental day, food was withdrawn from the metabolic cages at 15:00. Rats received injections (0.5 μl) of nesfatin-1 (50 pmol, 1–82; Phoenix Pharmaceuticals, Burlingame, CA, USA) or vehicle through the cannulae before the onset of the dark cycle. The protocol of microinjection and the doses selected were as described in our previous studies (Chen et al., 2012, 2015; Dong et al., 2014). Drug injections were administered at least 72 h apart.

Food was returned to the rats between 19:00 and 7:00 for continuous monitoring of meal patterns and water consumption, calculated as g/300 g body weight (Stengel et al., 2009a). The minimum meal amount was 0.5 g, and a new meal was considered based on an intermeal interval of 5 min.

Experiment 2: Effects of Nesfatin-1 in the LPBN on Long-Term Body Weight Gain

After completion of experiment 1, rats ($n = 24$) were individually housed and received 10 daily LPBN microinjections of nesfatin-1 or vehicle. The dose of nesfatin-1/vehicle was the same as in experiment 1. Body weights were recorded manually immediately before injections on day 1 and 24 h after each injection.

Experiment 3: Electrophysiological Effects of Nesfatin-1 on Glucose-Reactive Neurons in the LPBN

Rats ($n = 40$) were anesthetized with urethane (1.0 g/kg body weight, i.p.) and placed in a stereotaxic apparatus. Supplemental anesthetics were added when necessary throughout the procedure. A rectangular portion of bone was removed to expose the brain, and LPBN nuclei were localized. The exposed dura mater was carefully cleaned, and one side of the sinus was ligated and cut to give way for the microelectrode. The exposed area was covered with warm agar (3–4% in saline) to improve the stability for recording, and the rat was covered by a cotton pad to maintain body temperature during the surgery. The barrels of a four-barrel glass microelectrode (3–10 μm) (Chen et al., 2012; Dong et al., 2014) were filled with the following solutions: 0.5 M sodium acetate in 2% pontamine sky blue for recording, 5 mM glucose solution, 0.9% NaCl, and 1.5×10^{-8} M nesfatin-1 solution (Chen et al., 2012, 2015). The latter three microelectrodes were connected to a four-channel pressure injector (PM2000B; Micro Data Instrument, Inc., USA) to eject drugs by gas pressure (Chen et al., 2012; Dong et al., 2014).

LPBN neurons were localized at 8.8–9.0 mm caudal to bregma, 2.0–2.4 mm from midline, and a depth of 6.5–6.8 mm from the skull (Gaxinos and Watson, 2007). Glucose sensitive neurons in the LPBN were identified by their activity in response to the 5 mM glucose solution. After recovery, 0.9% NaCl was given as a control. At least 3 min were allotted to ensure that the baseline firing was stable, and 120 s of baseline data were collected before drug application. Next, the nesfatin-1 solution was applied and the alterations in firing rates were recorded. The drug was considered to exhibit significantly excitatory or inhibitory effects based on an increase or decrease in the firing rate, respectively, of at least 20%. A drug effect was calculated when the maximal alteration of frequency appeared within 50 s after drug administration. Only data from rats with injection sites in the correct location were included in the analyses (Supplementary Figure 2B).

Experiment 4: Influence of Nesfatin-1 in the Melanocortin System on Energy Balance

Effects of nesfatin-1 on feeding with SHU9119

Rats weighing 270–300 g underwent surgery as described in experiment 1 for the placement of unilateral guide cannulae and were divided into four groups ($n = 12$–14/group). Rats were individually housed and received daily injections (0.5 μl) through the cannulae into the LPBN for 10 days with 0.9% NaCl, nesfatin-1 (50 pmol), SHU9119 (250 pmol), or nesfatin-1 (50 pmol) + SHU9119 (250 pmol). Nesfatin-1 and SHU9119 (Sigma) were dissolved in 0.9% NaCl. Rats were weighed daily and food weights were recorded manually every hour for the first 6 h after injections and every 12 h thereafter (Stengel et al., 2009a). The doses of drugs were chosen based on the results of previous studies (Xu et al., 2015). The injection procedure was as described in experiment 1.

Adipose weight and morphometric analyse

Rats were sacrificed following the 10-day injection protocol. BAT from the interscapular region and unilateral white adipose tissue (WAT) from epididymal, inguinal, and perirenal regions were weighed. Portions of BAT were fixed in 4% formaldehyde in PBS (pH 7.4) for 24 h and embedded in paraffin before sectioning. Sections 10-μm thick were cut, were stained with Mayer's hematoxylin and eosin, and were imaged. Cell numbers were counted using Image J software (Unit area: 90×90 μm).

Western blot analysis

Remaining portions of BAT and WAT were frozen in liquid nitrogen and stored at −80°C until they were assayed for UCP1 protein levels. Adipose lysate proteins were subjected to 12% SDS-PAGE and electrotransferred to a polyvinylidene fluoride membrane (Millipore Corp., Billerica, MA, USA) for 2 h. After blocking, membranes were incubated overnight at 4°C in anti-UCP1 (ab10983; Abcam) and anti-beta-tubulin (2128S; Cell Signaling Technology) antibodies. Membranes were then incubated in secondary antibodies (ZB2301; ZSGB-BIO) for 1 h at room temperature. Antibody signals were developed using

Immobilon Western chemiluminescent substrate (Millipore, cat. no. WBKLS0100, 200 μl), and the intensities were analyzed using Image J software.

Statistical Analysis

Data are expressed as means ± standard error of the means (SEMs). Paired *t*-tests were used to compare firing rates before and after drug treatment. Data were analyzed using repeated-measures analyses of variance where appropriate. In all cases, $P < 0.05$ was considered significant.

RESULTS

Feeding Responses after Injection of Nesfatin-1 into the LPBN

We found that, compared with vehicle-treated counterparts, intra-LPBN microinjections of nesfatin-1 (50 pmol) significantly reduced nocturnal cumulative food intake beginning at 5 h (11.49 ± 0.7 vs. 8.52 ± 0.9 g, $P < 0.05$; **Figure 1A**), which was sustained through 12 h (18.70 ± 0.9 vs. 15.22 ± 1.3 g, $P < 0.05$; **Figure 1A**). Moreover, intra-LPBN microinjections of nesfatin-1 (50 pmol) had no effect on 12-h water intake (17.47 ± 1.6 vs. 15.72 ± 1.1 ml, $P > 0.05$; **Figure 1B**).

An analysis of feeding behaviors showed that during the first 6 h, nesfatin-1 had no effect on average meal number or meal size (**Figures 1C,D**). However, between 6 and 12 h postinjection, compared with those of rats receiving the vehicle, nesfatin-1 decreased the average meal sizes (2.83 ± 0.4 vs. 1.86 ± 0.7 g/meal, $P < 0.05$; **Figure 1E**) without affecting the meal numbers (**Figure 1F**).

Effect of Nesfatin-1 in the LPBN on Long-Term Body Weight Gain

To determine whether chronic administration of nesfatin-1 causes any changes in long-term body weight gain, rats received microinjections of either nesfatin-1 or saline into the LPBN once

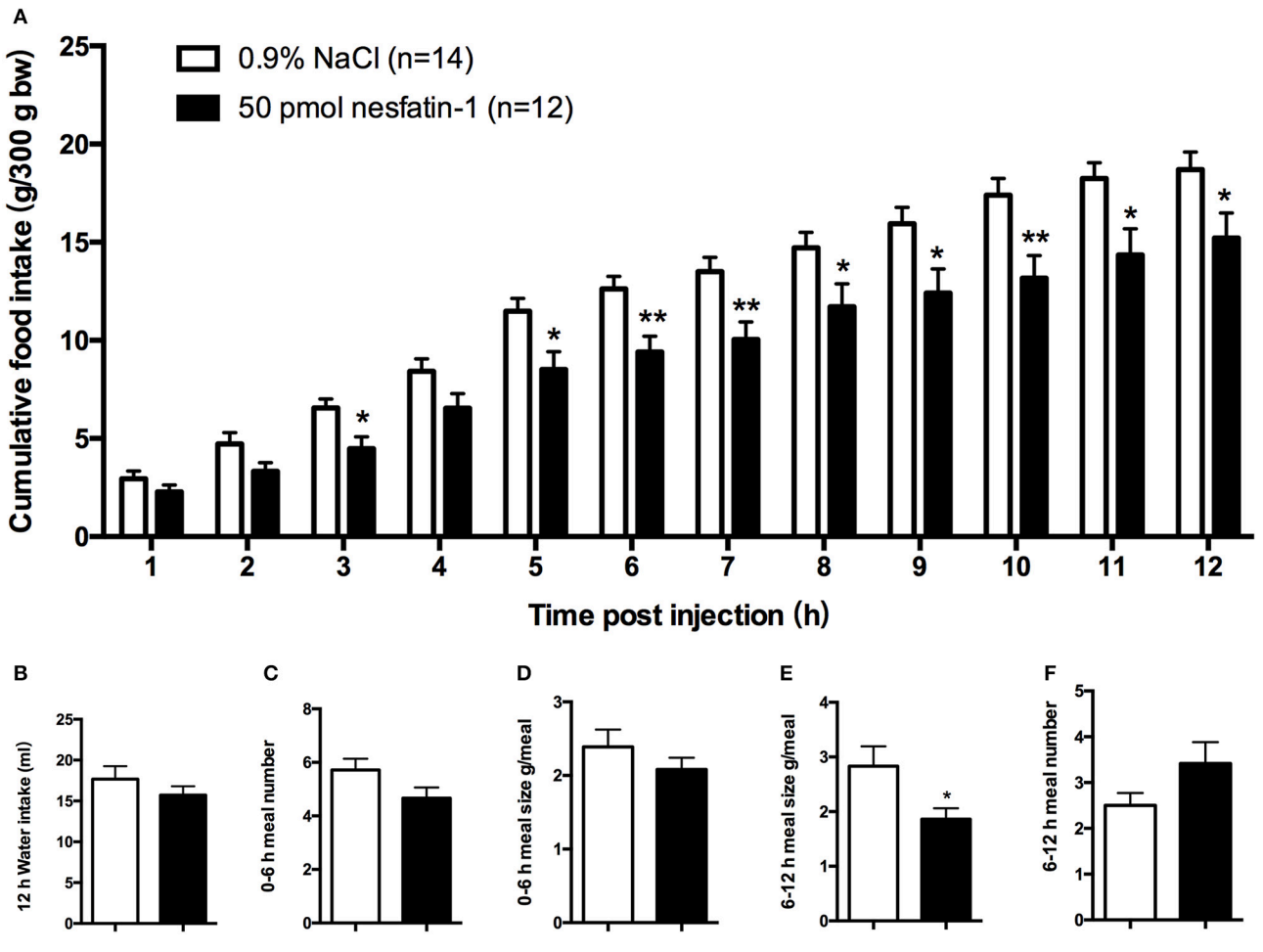

FIGURE 1 | Feeding responses to injections of nesfatin-1 in the LPBN. Nesfatin-1 injections (50 pmol) into the LPBN decreased cumulative food intake from 3 to 12 h after injection **(A)** without affecting 12-h water intake **(B)**. Nesfatin-1 (50 pmol/0.5 μl) or vehicle was microinjected into the LPBN of *ad libitum*-fed mice at the onset of the dark phase, and meal numbers at 0–6 h **(C)**, meal sizes at 0–6 h **(D)**, meal sizes at 6-12 h **(E)**, and meal numbers at 6–12 h **(F)** were assessed using an automated food intake-monitoring device. Bars represent the mean ± SEM of 12–14 rats/group. *$P < 0.05$, **$P < 0.01$ vs. saline vehicle-injected controls.

daily for 10 days. Compared with saline, nesfatin-1 reduced the amount of body weight gained beginning on day 4 (**Figure 2**). In the nesfatin-1-treated group ($n = 10$), the amount of body weight gained was reduced by 59.5, 57.6, 57.8, 50.6, 43.0, 37.3, and 42.0% compared with that in the saline-treated group ($n = 7$) on days 4, 5, 6, 7, 8, 9, and 10, respectively.

Effect of Nesfatin-1 on LPBN Glucose Sensitive Neurons *In vivo*

To investigate whether nesfatin-1 influences spontaneous discharging of LPBN neurons, electrophysiological recordings were made before, during, and after drug injections. Glucose excitatory and inhibitory neurons (GE and GI, respectively) were identified by injecting glucose through the microelectrode. Recordings from 46 neurons in the LPBNs of 44 rats after the application of 5 mM glucose revealed 9 (9/46; 19.6%) that were identified as GE neurons and 24 (24/46; 52.2%) that were identified as GI neurons. Of the 24 GI neurons examined, 14 were activated, 8 were depressed, and 2 failed to respond to nesfatin-1 (**Table 1**). Injection of nesfatin-1 increased significantly the firing rates of GI neurons from 2.46 ± 0.6 to 4.65 ± 0.8 Hz ($n = 11$, $P < 0.05$; **Figure 3A**). Of the 9 GE neurons, 3 were activated, 5 were depressed, and 1 failed to respond to nesfatin-1. Nesfatin-1 also decreased significantly the spontaneous firing rates of GE neurons from 3.26 ± 0.4 to 1.67 ± 0.5 Hz ($n = 4$, $P < 0.05$; **Figure 3B**).

Influence of SHU9119 on the Feeding Effects and Body Weight Changes of Nesfatin-1

The administration of SHU9119 abolished the inhibitory effect of nesfatin-1 on cumulative food intake at 4 h (7.28 ± 0.9 g vs. 3.66 ± 0.7 g, $P < 0.05$; **Figure 4A**) and 5 h (7.57 ± 1.3 g vs. 4.36 ± 0.9 g, $P < 0.05$; **Figure 4A**). Moreover, SHU9119 attenuated the effect of nesfatin-1 on the amount of body weight gained from day 4 to day 9 (day 4: 5.11 ± 1.2 vs. 10.67 ± 1.2 g, $P < 0.05$; day 9: 16.3 ± 2.5 vs. 25.3 ± 2.5, $P < 0.05$; **Figure 4B**). When compared with that in the saline-treated group, administration of SHU9119 significantly increased the amount of body weight gained only on day 5 (15.6 ± 2.6 vs. 23.3 ± 3.4 g, $P < 0.05$; **Figure 4B**).

We next investigated the effects of nesfatin-1 in the LPBN on adipose weight and UCP1 expression. To explore whether nesfatin-1 influences body weight by altering WAT distribution, we weighed inguinal, epididymal, and perirenal fat pads (**Figures 4C–E**). In addition, the expression of UCP1 in perirenal, epididymal and inguinal WAT was detected by Western blotting. The results show that there were no significant differences between the groups.

Effect of Nesfatin-1 in the LPBN and Involvement of the Melanocortin System on Increasing Brown Adipose Cell Numbers and UCP1 Protein Levels in bat

After 10 days of microinjecting nesfatin-1 into the LPBN, BAT samples appeared darker and redder (**Figure 5A**) and contained more cells (63.6 ± 1.3 vs. 49.7 ± 2.3 cells/unit area, $P < 0.05$; **Figures 5B,C**) than vehicle-treated animals. Long-term central injections of nesfatin-1 also increased the protein expression of UCP1 compared with than in vehicle-treated controls (2.39 ± 0.3 vs. 1.67 ± 0.3 AU, $P < 0.05$; **Figure 5D**).

However, administration of SHU9119 increased the number of lipid droplets and cells (79.8 ± 1.3 vs. 49.7 ± 2.3 cells/unit area, $P < 0.05$; **Figures 5B,C**) and decreased the expression levels of UCP1 (0.6 ± 0.1 vs. 1.67 ± 0.3 AU, $P < 0.05$; **Figure 5D**) in BAT compared with those in the vehicle-treated animals, as well as attenuated the nesfatin-1 effects compared with the group receiving nesfatin-1 only. Moreover, no significant differences were observed in the weights of BAT adipose pads (**Figure 5E**).

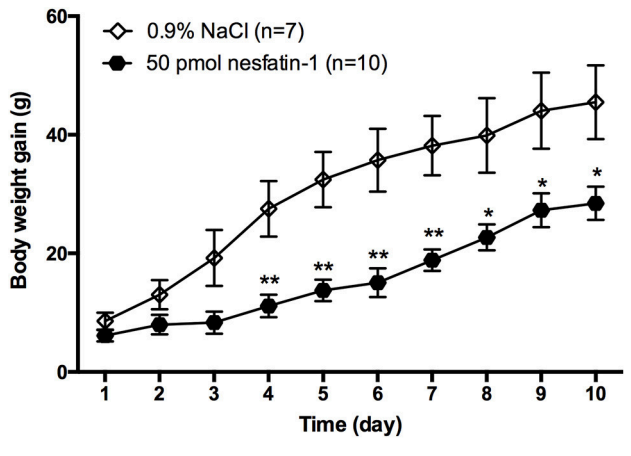

FIGURE 2 | Change in body weight gained after injections of nesfatin-1 for 10 days. Amount of body weight gained (increment from day 0) in rats after nesfatin-1 injections (50 pmol/0.5 μl daily). Data are mean ± SEM. *$P <$ 0.05, **$P < 0.01$ vs. saline vehicle-injected controls.

DISCUSSION

Although the role of nesfatin-1 in the hypothalamic nuclei and hindbrain in regulating food intake has been demonstrated (Oh et al., 2006), few studies have investigated the role of nesfatin-1 in the pontine nuclei. Recently, NUCB2/nesfatin-1 immunoreactivity was observed in the LPBN (Goebel et al., 2009, 2011a). Previous studies showed that administration of NUCB2/nesfatin-1 in the third ventricle or at the hindbrain level

TABLE 1 | Numbers of LPBN neurons responsive to glucose (5 mM) and nesfatin-1 (1.5×10^{-8}).

LPBN neurons ($n = 47$)	Nesfatin-1 response		
	Inhibited	Excited	Insensitive
GI neurons ($n = 24$; 52.2%)	8 (33.3%)	14 (58.3%)	2 (8.3%)
GE neurons ($n = 9$; 19.6%)	5 (55.6%)	3 (33.3%)	1 (11.1%)
Glucose-insensitive neurons ($n = 14$; 30.4%)	3 (21.4%)	0 (0%)	11 (78.6%)

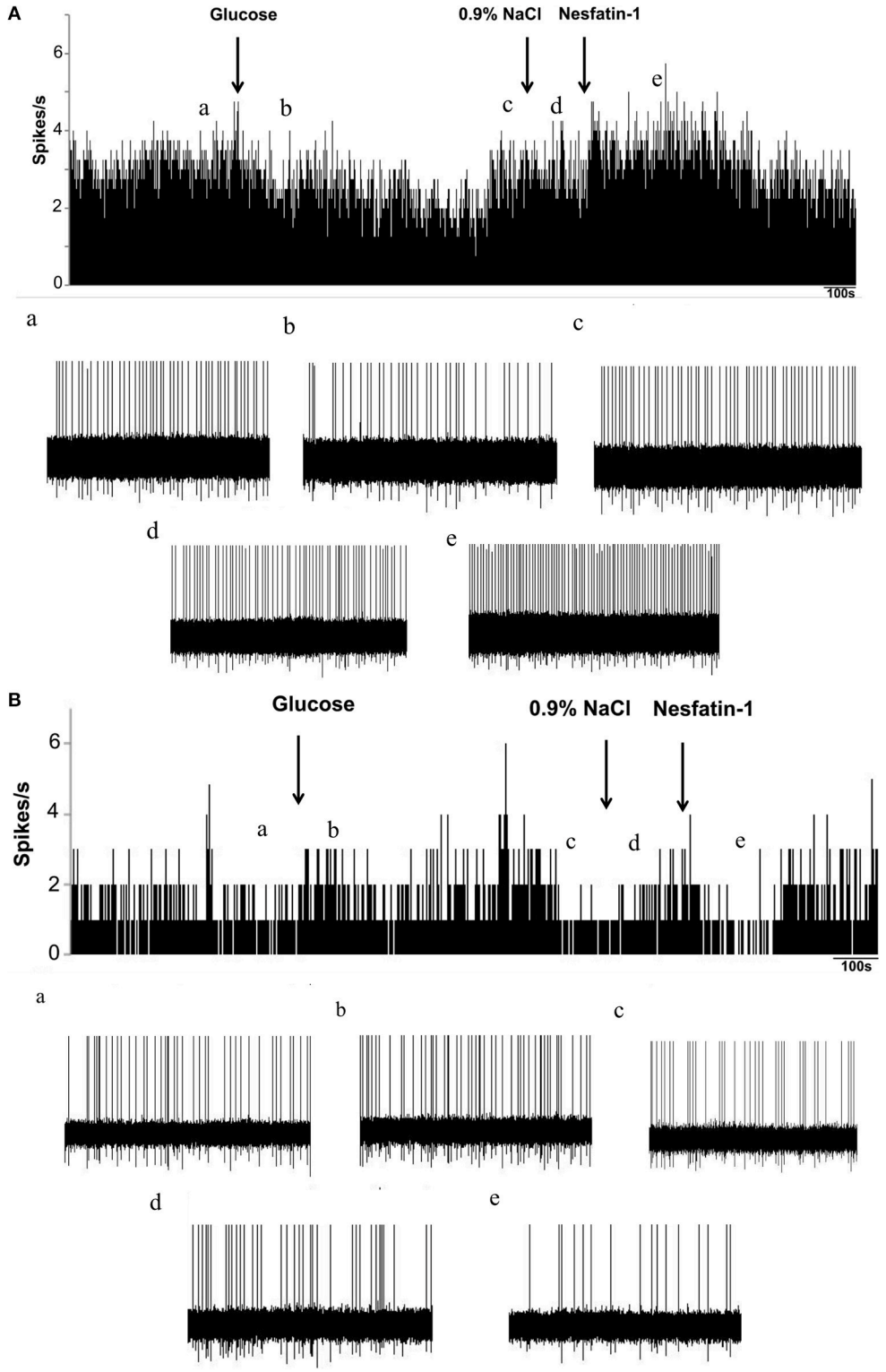

FIGURE 3 | Effects of nesfatin-1 on glucosensing neurons in the LPBN. (A) Representative stimulation of a GI neuron in the LPBN by nesaftin-1. The first arrow indicates addition of 5 mM glucose, the second arrow indicates the 0.9% NaCl-treated control, and the third arrow indicates nesfatin-1 (1.5×10^{-8} M) application. **(B)** Representative inhibition of a GE neuron by nesaftin-1. **(a)** Baseline before glucose application. **(b)** Firing rate after glucose application. **(c)** Firing rate recovered to baseline before 0.9% NaCl application. **(d)** Firing rate after 0.9% NaCl application and the baseline before nesfatin-1 application. **(e)** Firing rate after nesfatin-1 application.

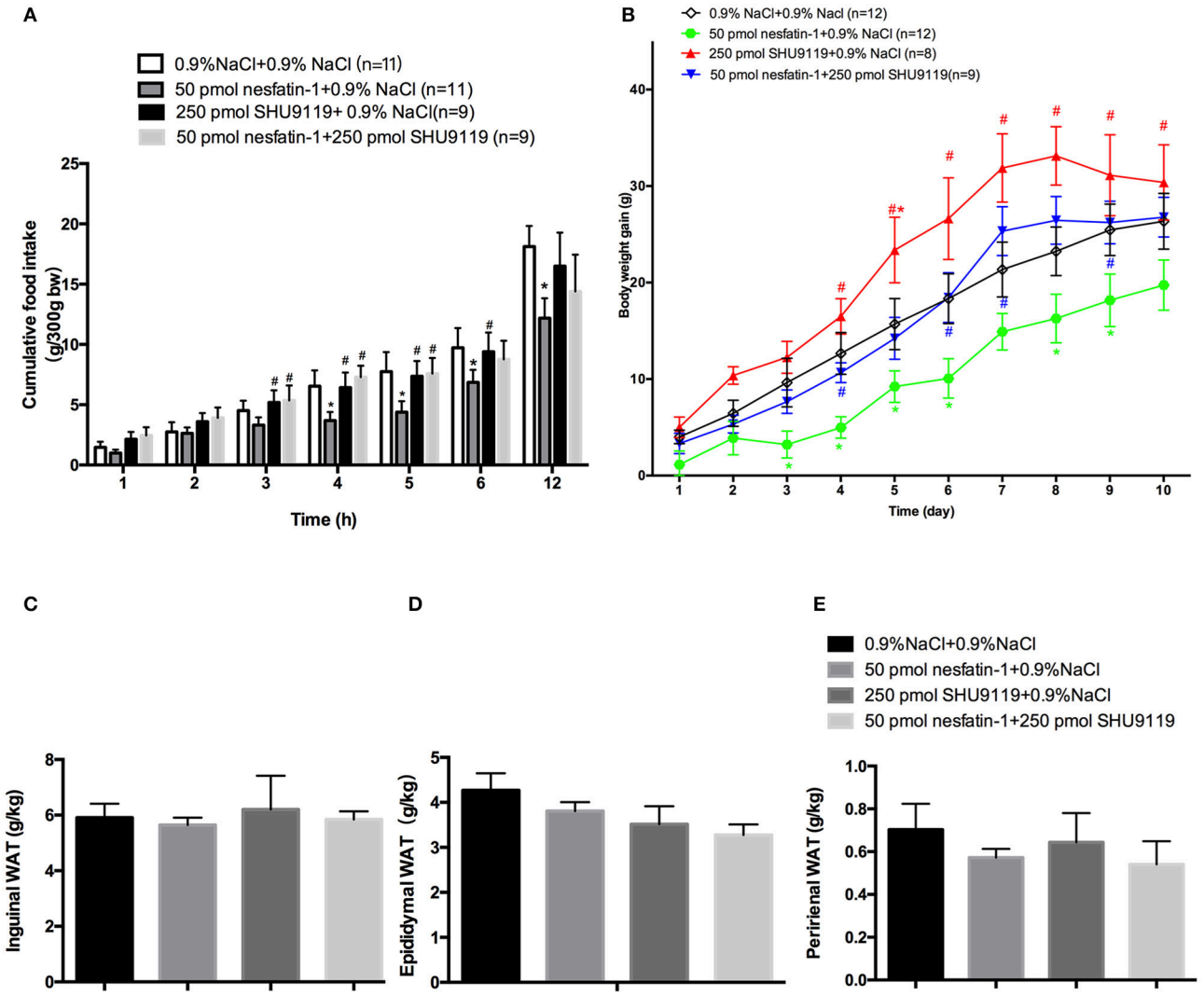

FIGURE 4 | Effects of SHU9119 on the feeding effects of nesfatin-1. (A) Effects of SHU9119 on nesfatin-1-induced nocturnal anorexia. Four groups of rats received microinjections (0.5 μl) into the LPBN at the onset of the dark cycle and cumulative food intake was recorded. **(B)** Effects of SHU9119 on nesfatin-1-induced body weight changes. Nesfatin-1-treated animals gained less weight than the other three groups. Rats were sacrificed after 10 days of central injections. **(C–E)** Unilateral WAT from epididymal, inguinal, and perirenal regions was weighed. Data represent the mean ± SEM of 9-11 rats/group. $^{*}P < 0.05$ vs. saline vehicle-injected controls; $^{\#}P < 0.05$ vs. nesfatin-1 group.

inhibits nocturnal feeding in rats (Oh et al., 2006; Stengel et al., 2009a). We extend these findings and show that nesfatin-1 in the LPBN induces anorexia between 5 and 12 h after administration, which is similar to that observed with injections in the forebrain. Nesfatin-1 also reduced nocturnal food intake by reducing meal sizes without affecting meal numbers, consistent with the results from a previous report (Goebel et al., 2011b).

Glucose sensitive neurons are located throughout the brain and integrate a variety of hormonal, metabolic, transmitter, and peptide signals involved in regulating energy homeostasis and other biological functions. Studies have shown that glucose sensitive neurons in the hypothalamus and brainstem are involved in glucose homeostasis and glucoprivic feeding (Ritter et al., 1998; Bonnet et al., 2013). There is also increasing interest in glucose sensitive neurons for their role in appetite regulation (Watts and Donovan, 2010). We previously investigated glucose sensitive neurons in hypothalamic nuclei (Chen et al., 2012) and the dorsal vagal complex (Dong et al., 2014) as a possible mechanism for the effects of nesfatin-1 on feeding behavior. As GLP-1 was reported to decrease food intake by increasing the firing rates of LPBN neurons (Richard et al., 2014), we investigated whether nesfatin-1 had a similar effect on a subpopulation of LPBN neurons that were found recently to be part of a glucose sensitive brain region (de Araujo, 2014). Garfield et al. demonstrated that LPBN cholecystokinin neurons are counterregulatory GI neurons whose activity does not impact the first 3 h of food intake (Garfield et al., 2014). In this study, we identified the majority of glucose sensitive

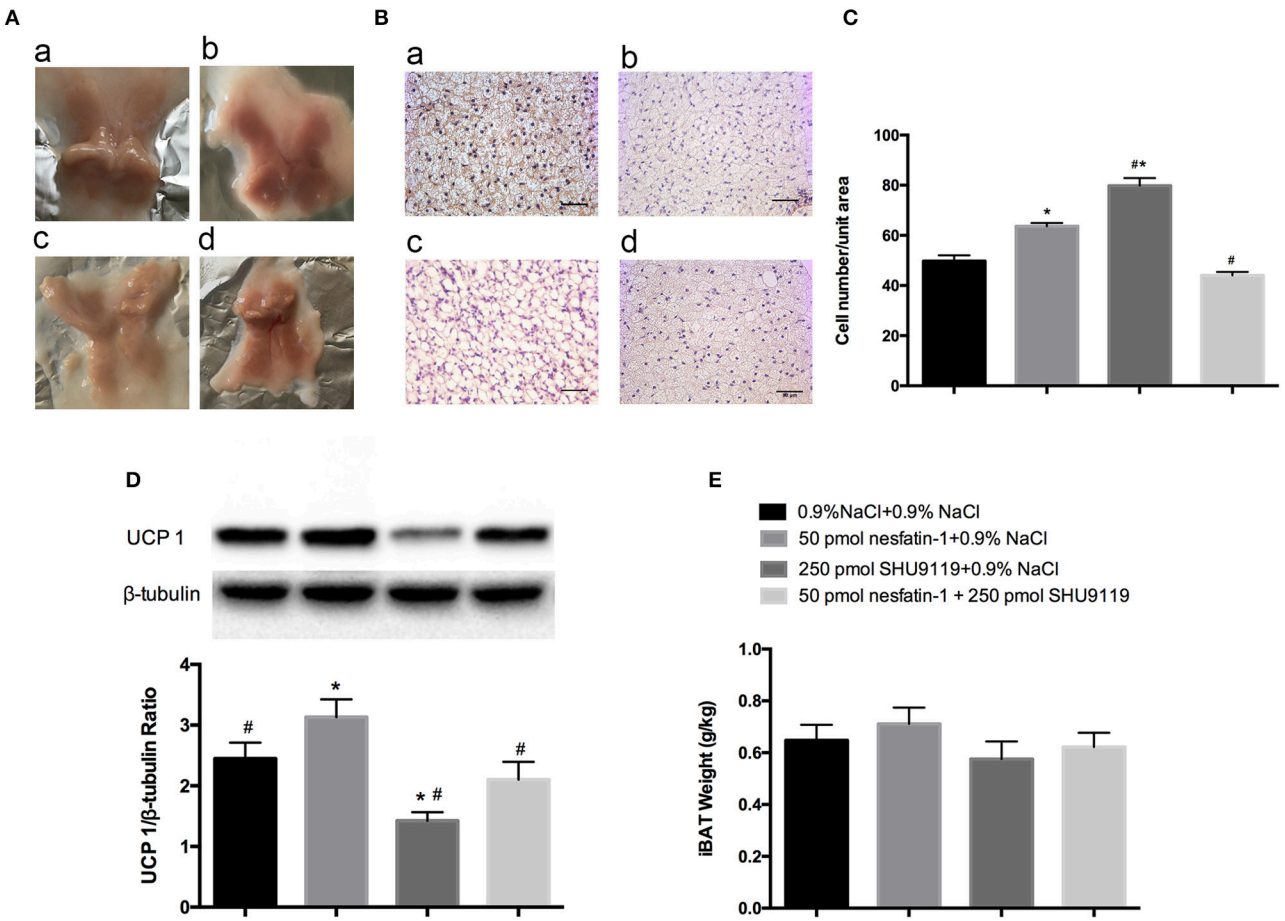

FIGURE 5 | BAT morphology and UCP1 protein analysis after 10 days of injections into the LPBN. Fresh interscapular BAT gross samples **(A)** and hematoxylin and eosin staining (200×) **(B)** after 10 days of microinjections into the LPBN; **(a)** 0.9% NaCl group, **(b)** 50 pmol nesfatin-1 group, **(c)** 250 pmol SHU9119 group, **(d)** 50 pmol nesfaint-1 + 250 pmol SHU9119 group. **(C)** Numbers of cell from the same unit areas were counted and recorded. UCP1 protein expression levels in **(D)** and weights of **(E)** BAT were measured. Data represent the mean ± SEM of 7–12 rats/group. $^{*}P < 0.05$ vs. saline vehicle-injected controls; $^{\#}P < 0.05$ vs. nesfatin-1 group.

neurons in the LPBN as GI neurons, of which 58.3% were excited by nesfatin-1. Thus, nesfatin-1 recapitulates the effect of glucose deprivation on GI neurons and inhibits feeding too. In addition, Wu et al. demonstrated that loss of GABAergic inhibition from agouti-related peptide-producing neurons leads to abnormal activation of the parabrachial nucleus, which in turn inhibits feeding (Wu et al., 2012). Therefore, the mechanism for nesfatin-1's anorexigenic effect is still not completely clear. Further investigation is needed to clarify the final pathways and neurotransmitters impacting the glucose sensitive neurons in the LPBN.

In the central nervous system, nesfatin-1 is thought to be involved both in glucose sensing and in the control of glucose metabolism (Dore et al., 2017). Surprisingly, the areas in the LPBN that are immunoreactive for NUCB2/nesfatin-1 (Goebel et al., 2009, 2011a) and 2-deoxyglucose or insulin-induced c-fos (Garfield et al., 2014) partially overlap. Bonnet et al. (2013) showed that insulin-activated neurons express nesfatin-1 in the hypothalamus and the dorsal vagal complex, supporting the hypothesis that glucoprivation stimulates nesfatinergic neurons. However, studies from Su et al. and Li et al. showed no effect of central nesfatin-1 on blood glucose, glucose tolerance, or insulin sensitivity (Su et al., 2009; Li et al., 2013). Nevertheless, most GI neurons in the LPBN are also nesfatin-1 responsive. Anatomical studies have shown that the LPBN contains at least seven separate subnuclei that can be distinguished by morphology, spatial clustering, and afferent and efferent connectivities (Hayward and Felder, 1999). However, our electrophysiological studies were not restricted to a specific subarea within the LPBN. Further investigation is still needed to determine whether nesfatin-1 functions as a paracrine or autocrine hormone. The identification of a nesfatin-1 receptor(s), as well as subsequent structural-functional analyses, would certainly aid in defining the precise physiological roles of nesfatin-1.

We detected a delayed onset of anorexia after fasting that was induced by nesfatin-1, which corresponds with electrophysiological data (excited GI neurons and inhibited GS neurons) from the PVN (Chen et al., 2012) and the

LPBN. The anorexia-related actions of nesfatin-1 result from oxytocin and melatonin signaling in several hypothalamic and medullary anorexigenic pathways (Oh et al., 2006; Maejima et al., 2009; Stengel et al., 2009b; Yosten and Samson, 2009). The LPBN, PVN, and solitary tract nucleus contain oxytocin and pro-opiomelanocortin neurons. Satiety signals from the gut ascend via the afferent vagal nerve to the solitary tract nucleus and continue to the forebrain directly and also indirectly via a relay in the LPBN (Herbert et al., 1990; Karimnamazi et al., 2002; Schwartz, 2009; Grill and Hayes, 2012). Melanocortin-4 receptor-expressing neurons in the PVN project to the LPBN (Garfield et al., 2015), and cholecystokinin-expressing GI neurons in the LPBN target steroidogenic-factor 1-expressing neurons in the ventromedial nucleus of the hypothalamus (Alhadeff et al., 2014a). Although we still do not know the details regarding the neuronal circuitry of the effects of nesfatin-1 in the LPBN, it is possible that nesfatin-1 in the parabrachial nucleus works cooperatively with the PVN pathway.

By administering SHU9119 (250 pmol), we confirmed that nesfatin-1 is involved in the melatonin system, as was found with intracerebroventricular and third ventricle injections in previous studies (Oh et al., 2006; Yosten and Samson, 2009). The evidence also suggests that the anorexigenic action of nesfatin-1 is activated by a hypothalamic-pontine oxytocin-pro-opiomelanocortin-α melanocyte-stimulating hormone-melanocortin 3/4 receptor-signaling pathway (Stengel et al., 2013). In addition to controlling feeding, hypothalamic and hindbrain melanocortin receptors also contribute to energy expenditure (Skibicka and Grill, 2009). The thermogenic actions of nesfatin-1 were first demonstrated by increased core body temperatures after intracerebroventricular administration of nesfatin-1 (25 pmol) (Konczol et al., 2012). Moreover, Wernecke et al. (2014) demonstrated that nesfatin-1 reduces food intake and increases energy expenditure. A possible mechanism of nesfatin-1-induced thermogenesis most likely depends on sympathetic nervous system activation of BAT. The sympathetic nervous system utilizes melanocortin to signal to BAT from the LPBN (Skibicka and Grill, 2009). Nesfatin-1 can also activate the sympathetic nervous system (Yosten and Samson, 2009) and colocalizes with pro-opiomelanocortin (Brailoiu et al., 2007), which suggests that nesfatin-1 might be involved in melanocortin-regulated energy expenditure. In this study, we detected an elevated level of UCP1 in BAT after long-term nesfatin-1 administration, which was abolished by SHU9119. Although we did not measure BAT temperatures, the elevated level of UCP1 protein supports our hypothesis. Nevertheless, nesfatin-1 appears to act as an important negative regulator of energy balance.

A change in body weight can result from altering the balance of food intake and energy expenditure. In fact, we also measured food intake discontinuously during the long-term study after injection of nesfatin-1. On the 3rd day, nesfatin-1's inhibitory effect on food intake was similar to that of the 1st day (as shown in the Supplementary Figure 3). On the 5th day, nesfatin-1 only inhibited cumulative food intake in 6 h (Supplementary Figure 4). On the 7th day, the inhibitory effect of nesfatin-1 on food intake even disappeared (Supplementary Figure 5). Moreover, the body weight gain also showed no difference on the same day (as shown in **Figure 4B**), which indicated the suppression of food intake might account mainly for the reduction of body weight gain before the 7th day. The food intake inhibiting effect of nesfatin-1 seemed to be lost after 1 week of treatment, maybe due to the compensatory effect of other feeding related peptides. However, the body weight gain still kept reductive tendency till the 10th day, which suggested that the nesfatin-1 thermogenic effect might be responsible for the lower body weight gain during the last few days of our long-term treatment experiment. Compared with nesfatin-1's effect on food intake, its participation in regulating energy expenditure is less well investigated. Intracerebroventricular injections of nesfatin-1 reduce the duration of nocturnal food intake for 2 days and raise body core temperatures (Konczol et al., 2012) and significantly stimulate renal sympathetic nerve activity (Tanida and Mori, 2011), which also acts as an outflow of energy expenditure. Our previous study also showed that nesfatin-1 stimulated free fatty acid utilization in skeletal muscle in T2DM mice (Dong et al., 2013). In the present study, activation of BAT was observed as one of the energy output pathways. Therefore, both inhibition of food intake and promotion of energy expenditure contributed to the reduction of body weight gain after injection of nesfatin-1 in LPBN.

CONCLUSION

Nesfatin-1 in the LPBN is involved in energy homeostasis by inhibiting food intake, modulating the excitability of glucose sensitive neurons, and acting on the melanocortin system to enhance UCP1 expression in BAT.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JD, ZJ, JY, and XC. Performed the experiments: JY, XC, KS, GW, XH, YZ, and DZ. Analyzed the data: JY and XC. Wrote the paper: JY and XC. Edited the manuscript: JD, XC, JY, ZJ, and PC. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00235/full#supplementary-material

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REVIEW
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Food for Mood: Relevance of Nutritional Omega-3 Fatty Acids for Depression and Anxiety

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The central nervous system (CNS) has the highest concentration of lipids in the organism after adipose tissue. Among these lipids, the brain is particularly enriched with polyunsaturated fatty acids (PUFAs) represented by the omega-6 (ω6) and omega-3 (ω3) series. These PUFAs include arachidonic acid (AA) and docosahexaenoic acid (DHA), respectively. PUFAs have received substantial attention as being relevant to many brain diseases, including anxiety and depression. This review addresses an important question in the area of nutritional neuroscience regarding the importance of ω3 PUFAs in the prevention and/or treatment of neuropsychiatric diseases, mainly depression and anxiety. In particular, it focuses on clinical and experimental data linking dietary intake of ω3 PUFAs and depression or anxiety. In particular, we will discuss recent experimental data highlighting how ω3 PUFAs can modulate neurobiological processes involved in the pathophysiology of anxiety and depression. Potential mechanisms involved in the neuroprotective and corrective activity of ω3 PUFAs in the brain are discussed, in particular the sensing activity of free fatty acid receptors and the activity of the PUFAs-derived endocannabinoid system and the hypothalamic–pituitary–adrenal axis.

Keywords: omega-3 fatty acid, endocannabinoids, HPA axis, nutrient sensing, mood disorders, anxiety, depression, DHA

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INTRODUCTION

Since the discovery of omega-3 (ω3) PUFAs in 1929 by George Burr and Mildred Burr (Burr and Burr, 1929; Spector and Kim, 2015), research on ω3 PUFAs became an appealing topic ranging from their role in cardiovascular risk to more recently neuropsychiatric pathologies such as depression and anxiety, cognitive decline or neurodegenerative diseases (Bazinet and Layé, 2014; Joffre et al., 2014; Coulombe et al., 2017). The relevance of lipids in brain function is illustrated by the fact that the CNS has the highest concentration of lipids in the organism after the

Abbreviations: 2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; AC, adenylyl cyclase; AEA, ethanolamides anandamide; ALA, alpha-linolenic acid; CA1, cornu ammonis 1; cAMP, cyclic adenosine monophosphate; CB1R, cannabinoid receptor 1; CNS, central nervous system; COX, cyclooxygenase; CRH, corticotrophin-releasing hormone; CSDS, chronic social defeat stress; CYP450, cytochrome P450; DHA, docosahexaenoic acid; DHEA, docosahexaenoyl ethanolamide; DPA, docosapentaenoic acid; eCB, endocannabinoid; EPA, eicosapentaenoic acid; FADS, fatty acid desaturases; FFAR, free fatty acid receptors; FST, forced swimming test; GABA, gamma-aminobutyric acid; GPCR, G-protein-coupled receptor; GPR120, G-protein-coupled receptor 120; GPR40, G-protein-coupled receptor 40; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; IL, interleukin; LA, linoleic acid; LC, long chain; LOX, lipoxygenases; LPS, lipopolysaccharide; NAc, nucleus accumbens; PFC, prefrontal cortex; PPAR, peroxisome proliferator-activated receptor; PTSD, post-traumatic stress disorders; PUFAs, polyunsaturated fatty acids; RXR, retinoid X receptor; SPMs, specialized pro-resolving lipid mediators; SSRI, selective serotonin reuptake inhibitor; TNFα, tumor necrosis factor α; WHO, World Health Organization.

adipose tissue (50–60% of the dry weight of the brain; Sastry, 1985). Among these lipids, the brain is particularly greedy for PUFAs from the ω6 and ω3 PUFAs families, in particular the LC PUFA (AA, 20:4n-6) and (DHA, 22:6n-3), respectively (Sastry, 1985). In the Human brain, DHA accounts for 10 to 15% of the total fatty acids (saturated, monounsaturated and PUFAs) in both males and females (McNamara et al., 2007, 2008b). This makes PUFAs indispensable to the normal development and function of the CNS (Makrides and Gibson, 2000; Innis, 2007; Bazinet and Layé, 2014). One hypothesis explaining this abundance in brain tissue is that *Homo sapiens* in Paleolithic settled around the lakes and seas where access to foods rich in ω3 PUFAs is easy (Bradbury, 2011). It is generally considered that humans evolved on a diet with a ratio of ω6 to ω3 PUFAs equal approximately to 1. During the industrial era, the rapid expansion of Western countries has been associated with drastic changes in the ω6/ω3 PUFAs content of the diet. This is reflected in large quantities of ω6 PUFA-containing foods and smaller amounts of ω3 PUFA-rich foods leading to Western diet being typically poor in ω3 PUFAs. In addition, the intake of saturated fats from lard and butter has been replaced by plant-based PUFAs based on recommendations from health agencies (Gibson et al., 2011). As a result, the use of oils such as sunflower oil which are mostly high in (LA, the precursor of AA) and low in a-linolenic acid (ALA, the precursor of DHA) leads to a marked increase in LA intake. In mammals, LA and ALA cannot be synthesized *de novo* and need to be provided through the diet (Simopoulos, 1991; Gibson and Makrides, 2001). These essential PUFAs are metabolized into LC PUFAs using the same enzymatic pathway, meaning that LA and ALA are in competition for endogenous conversion to their respective LC forms AA, and DHA (**Figure 1**) but also for their entry into the brain (Bazinet and Layé, 2014). Of importance, ALA bioconversion into DHA through several cycles of elongation (ELOVLs) and desaturation (Δ5 and Δ6 desaturases) is in the range of 0.05% (Burdge et al., 2003) to 4% (Emken et al., 1994) and might not be sufficient to cover brain needs. This led to the recommendation of dietary intake of oily fish rich in the LC ω3 PUFAs DHA and EPA (Tejera et al., 2016). Overall, western diets which are rich in LA (coming from vegetable oils rich in LA) and poor in ALA and DHA (coming from fat fish, sea food or certain algae) have created "a conditional essentiality for ω3 PUFAs" as previously described by Cunnane (2003) and Gibson et al. (2011). Indeed, the amount of LC ω3 PUFAs needed to compensate this lack in western diet is likely to increase, which is not sustainable in the actual context of fish stock decline (Fernandes and Cook, 2013).

The reduced dietary supply of ω3 PUFAs to the brain is associated with many brain diseases, including depression and anxiety disorders (see review from Müller et al., 2015). Epidemiological studies have linked low ω3 PUFAs dietary intake with the prevalence of depression in the general population (Hibbeln, 1998). Clinical studies further revealed that subjects diagnosed with depression or anxiety display significant lower levels of ω3 PUFAs and higher ratio of ω6 to ω3 PUFAs in the blood and in the brain (Green et al., 2006; McNamara and Liu, 2011; Parletta et al., 2016). Supporting clinical observations, preclinical studies conducted in rodents showed that ω3 PUFA deficient diet consumption induces depressive- and anxiety-like symptoms as well as abnormal social behavior in adult offspring (Lafourcade et al., 2011; Larrieu et al., 2012, 2014, 2015; Bondi et al., 2014). Importantly, the use of dietary animal models has been crucial to study the neurobiological mechanisms underlying the alteration of emotional behaviors following decreased bioavailability of ω3 PUFAs in the brain. In this review, we first discuss clinical and pre-clinical evidence of the importance of ω3 PUFAs in anxiety and depressive disorders as well as the rationale for evaluating baseline levels of ω3 PUFAs prior to starting nutritional intervention studies. Then we describe mechanisms linking ω3 PUFAs and emotional behaviors disturbance, especially the sensing activity of FFAR, the eCB system, glucocorticoids as well as neuroinflammatory pathways.

THE ROLE OF ω3 PUFAs IN DEPRESSION AND ANXIETY DISORDERS

Clinical and Epidemiological Evidence Linking ω3 PUFAs, Depression and Anxiety

Several clinical and epidemiological studies highlighted the link between mood disorders and blood and/or cellular membrane PUFAs content (reviewed in Müller et al., 2015). These observations led to the "phospholipids hypothesis" according to which PUFAs are possible aetiological factors in the development

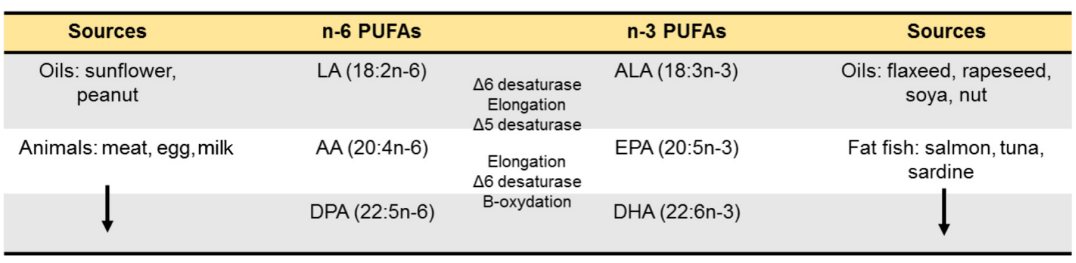

FIGURE 1 | Long-chain PUFAs synthesis. Essential fatty acids precursors of n-6 and n-3 PUFAs are provided by food. Once in the livers, they are metabolized into long-chain PUFAs using a series of desaturations and elongation machinery. The newly synthetized long-chain n-6 PUFAs are AA (20:4n-6) and DPA (22:5n-6) and the long-chain n-3 PUFAs are DHA (22:6n-3) and DPA (22:5n-3).

of depressive disorders (Hibbeln and Salem, 1995). Indeed, subjects diagnosed for anxiety and depressive disorders show lower ω3 PUFAs and higher ratio of ω6 to ω3 PUFAs in their blood and brains compared to healthy subjects matching for age and sex (Adams et al., 1996; Maes et al., 1996; Edwards et al., 1998a,b; Tiemeier et al., 2003; Frasure-Smith et al., 2004; Green et al., 2006; McNamara et al., 2007; McNamara and Liu, 2011; Parletta et al., 2016). EPA (20:5 n-3) concentration (Adams et al., 1996; Green et al., 2006; Liu et al., 2013) as well as DHA concentration (Edwards et al., 1998a; Frasure-Smith et al., 2004; Green et al., 2006; McNamara and Liu, 2011; Liu et al., 2013; Otoki et al., 2017) are decreased in the membrane of erythrocytes and in the plasma of patients suffering from unipolar depression, seasonal winter affective disorder or social anxiety disorders (Adams et al., 1996; Green et al., 2006). A recent study showed that the AA:EPA ratio in the blood is positively correlated with illness duration in patients diagnosed with major depression (Scola et al., 2018). A meta-analysis work from Lin et al. (2010) supports these observations by showing significant low levels of EPA, DHA, and total ω3 PUFAs among 3,318 depressed patients. In some of these studies, the severity of the depressive and anxious symptoms is negatively correlated with the concentration of the total ω3 PUFA levels in the blood (Adams et al., 1996; Maes et al., 1996; Edwards et al., 1998a; Green et al., 2006; Liu et al., 2013). Post-mortem studies report reduced levels of DHA in the PFC of patients diagnosed with major depression, bipolar disorders or committing suicide (McNamara et al., 2007, 2008a, 2013). In pregnant women, for whom the risk of ω3 PUFAs deficiency is relatively high as they provide DHA to the fetus, nearly 10% experience post-partum depression (Markhus et al., 2013). Yet, no clear consensus exist as to whether low ω3 PUFAs and high ω6 PUFA levels in the blood are linked to the development of post-partum depression (Hibbeln, 2002; Parker et al., 2015). These observations are not limited to depression as decreased ω3 PUFAs (DHA) levels in erythrocytes and PFC have also been found in patients suffering from PTSD (de Vries et al., 2016). As decreased ω3 PUFAs status is associated with several forms of depression and stress disorders, the understanding of the origin (dietary or genetic) of the decreased bioavailability of DHA or EPA is of high interest.

The rationale for identifying baseline nutritional ω3 PUFAs status comes from one hypothesis advanced by researchers that weak food supply in ω3 PUFAs might be a risk factor of the development of depression. As fish is the main source of LC ω3 PUFAs, several epidemiological studies investigating putative associations between major depressive disorder and fish consumption were conducted in various countries (Finland, New Zealand, France, Northern Ireland, Norway or Netherlands) (Tanskanen et al., 2001; Silvers and Scott, 2002; Timonen et al., 2004; Barberger-Gateau et al., 2005; Kamphuis et al., 2006; Appleton et al., 2007; Raeder et al., 2007; Colangelo et al., 2009). Subjects having low fish consumption (lower than once per week, including seafood) present high scores of depression (Timonen et al., 2004; Barberger-Gateau et al., 2005). A transnational ecological study conducted on a large cohort including individuals from different countries highlighted a strong negative correlation between fish consumption and the prevalence of depression (Hibbeln, 1998). Indeed, this epidemiological study of great width (170,000 individuals) revealed that individuals from Asian countries like Japan, Korea, and Taiwan, who are the biggest fish consumers, suffer relatively little from major depression. This observation can appear counterintuitive as Japan experiences a high rate of suicide while the depression rate is thought very low (WHO, 2015). Such a high rate of suicide has been associated to cultural factors (idealization of suicide, acceptability, etc.) including aging society (Saito et al., 2013), divorce and unemployment (Yamauchi et al., 2013). On the contrary, Western countries like New Zealand, Canada, United States, Germany, or France are part of the countries that consume less fish with high prevalence to develop depression. These data suggest that fish consumption is conversely correlated with the development of depression.

To PUFA dietary intake consideration in decreasing ω3 PUFAs bioavailability in depression, one must add the genetic variation of the FADS, an enzyme which converts PUFA precursors into LC-PUFAs (EPA, DHA, and AA) (Koletzko et al., 2011; Mathias et al., 2014; Park et al., 2015). Indeed, inter-individual variability in red blood cells DHA and AA levels is explained by FADS polygenes (71 and 53%, respectively) (Lemaitre et al., 2008). FADS genotypes influence DHA amounts in red blood cells of pregnant women independently of dietary effects (Koletzko et al., 2011). Children carrying FADS minor allele have lower DHA levels in erythrocyte, with no behavioral outcomes (Jensen et al., 2014). However, the link between FADS haplotype and the risk of developing neuropsychiatric disorders (schizophrenia or depression) is weak (Fallin et al., 2004; Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, 2011). A study conducted in patients with major depression found no association between FADS single nucleotide polymorphisms and major depression (Sublette et al., 2016). Recently, a study suggested that genetic variation in the FADS gene influences the ω6/ω3 PUFAs ratio which appears to be associated with major depression (Cribb et al., 2017). To our knowledge, no study has so far linked brain DHA level to FADS genotype. Overall, in addition to dietary composition of PUFAs, FADS genetic variation should be considered in the LC-PUFAs status and the pathophysiology of depression and stress.

Taken together, these clinical observations raise a crucial question: Is there a causal link between the contents of ω3 PUFAs in the blood/brain and depressive/anxiety disorders? If so, are the low levels of ω3 PUFAs the cause or the consequence of these affective disorders? These relations of causalities were approached by nutritional interventions in Humans which are the object of the following section, first in depression, then in PTSD and stress disorders.

Dietary ω3 PUFAs Supplementation, Depression, and PTSD in Humans

The results from LC ω3 PUFA nutritional interventions carried out among patients with depressive disorders are heterogeneous as recently reviewed elsewhere (Bozzatello et al., 2016; Saunders et al., 2016). Some studies conducted on patients suffering from major depression without an antidepressant treatment

do not show significant effects of ω3 PUFAs supplementation (Marangell et al., 2003; Freeman et al., 2008; Rees et al., 2008; Mischoulon et al., 2015), whereas others reveal a beneficial effect (Su et al., 2003; Nemets et al., 2006; Jazayeri et al., 2008). A 16-week dietary supplementation with EPA + DHA did not prevent maternal depressive symptoms (Vaz et al., 2017). These discrepancies are reflected by meta-analysis. Indeed, some found that EPA and DHA can reduce depressive symptoms (Kraguljac et al., 2009; Appleton et al., 2010; Sublette et al., 2011; Grosso et al., 2014) while other found no effect (Appleton et al., 2015). Mixed results of clinical trials could be attributed not only to the heterogeneity in clinical trials and design, but also to the quantity and quality of the PUFA used, including the EPA:DHA ratio, trial duration, the type of placebo (PUFA or other fatty acids) and to the concomitant use of medication and baseline symptom severity. Recently, EPA, rather than DHA, has been suggested to mediate the beneficial effect of ω3 PUFAs supplementation in patients diagnosed with major depression DHA (Martins, 2009; Martins et al., 2012; Grosso et al., 2014; Hallahan et al., 2016). Indeed, several studies using ethyl-EPA (from 1 to 2 g/day) reported a beneficial effect in patients with major depression and resistant to anti-depressant (Peet and Horrobin, 2002) or recurrent unipolar depression (Nemets et al., 2002). In addition, EPA-rich formulation with no DHA is more effective than DHA-rich supplements in major depression (Sublette et al., 2011). A meta-analysis aiming at investigating the beneficial role of EPA or DHA supplementation in major depressive disorder found that EPA is more effective than DHA (Grosso et al., 2014). The beneficial effect of EPA has been recently corroborated by a new meta-analysis (Mocking et al., 2016). EPA efficiency could be linked to its conversion into DHA by elongase leading to increased DHA brain bioavailability and decreased LC ω6 PUFAs production (Ganança et al., 2017). Indeed, as the conversion of EPA into DHA compete with the production of n-6 DPA from AA by using the same enzymatic pathway (i.e., FADS and elongases), the supplementation of EPA can simultaneously lead to an increase in DHA and a decrease in n-6 DPA levels that can subsequently improve mood. However, further studies with larger and more homogeneous samples are required to confirm these effects.

In addition, it has been suggested that depressive patients who display low levels of ω3 PUFAs may rather benefit of the LC ω3 PUFAs supplementation (Carney et al., 2016; Messamore and McNamara, 2016). Despite recent advances in understanding the pathophysiology of major depression, approximately 30% of patients remain refractory to multistep antidepressant treatments (Rush et al., 2006a,b). One explanation of this finding could come from individual differences in baseline levels of ω3 PUFAs. To support this idea, a study recently showed that high baseline levels of EPA and DHA in red blood cells of depressive patients predict favorable depression outcomes in patients receiving ω3 PUFAs supplements (Carney et al., 2016). In addition, among patients treated but resistant to antidepressants such as the (SSRI; e.g., Fluoxetine, Paroxetine), the severity of the symptoms of depression decreased in the group supplemented in ω3 PUFAs (Nemets et al., 2002; Peet and Horrobin, 2002; Su et al., 2003; Jazayeri et al., 2008; Gertsik et al., 2012; Zimmer et al., 2013; McNamara et al., 2014; Mocking et al., 2016). This strategy can be highly relevant as resistance to treatment is observed in a large proportion of patients (40%) (Brunoni et al., 2009; Shelton et al., 2010) and it suggests that dietary ω3 PUFA intake may improve antidepressant response.

ω3 PUFAs dietary supplementation has also been used in stress disorders. Stress is a well-known major risk factor for the development of depression or PTSD. One study aimed at investigating the effect of ω3 PUFAs supplementation in chronically work-stressed individuals and found no significant treatment effect for EPA after 12 weeks of supplementation on the Perceived Stress Scale scores (Bradbury et al., 2017). On the contrary, a placebo-controlled trial of ω3 PUFAs supplements in patients suffering from PTSD revealed that EPA but not DHA (Matsuoka et al., 2015) levels were inversely correlated with PTSD severity suggesting the potential efficacy of EPA rather than DHA for minimizing PTSD symptoms (Matsuoka et al., 2016). Regarding ω3 PUFAs supplementation in the prevention of anxiety, a study conducted by Yehuda et al. (2005) has investigated whether administration of a cocktail of ω3 PUFAs (90 mg of ALA/day) and ω6 PUFAs (360 mg of LA/day) over 3 weeks in students could improve anxiety induced by the university examinations. These authors highlighted an improvement of several symptoms (appetite, mood, concentration, and fatigue) compared to the placebo group. These improvements are associated with a decreased level of salivary cortisol (Yehuda et al., 2005). Despite the low dose of ALA used in this study (90 mg/day) as compared to nutritional intake in the general population (1–2 g/day), this 10% increase was sufficient to improve symptoms. In addition, ALA conversion in EPA is determined by the amount of ALA in the diet (i.e., higher in the plasma phospholipid pool when ALA is low) (Goyens et al., 2006). Thus, the effectiveness of ALA in Yehuda et al.'s (2005) study could be linked to EPA. Moreover, students having received a supplementation with DHA and EPA during 12 weeks present a reduction of 20% of anxiety symptoms compared to the students treated with a placebo (Kiecolt-Glaser et al., 2011). In students treated with ω3 PUFAs supplementation, an increase in plasma concentration of DHA and EPA were observed as of the third week of treatment. Lastly, the increase in DHA and EPA was negatively correlated with a reduction in anxiety symptoms.

In conclusion, these observations further support the role of PUFAs metabolism as an important mechanism in depression and anxiety disorders treatment. This brings new insight to personalized PUFAs formulation as a novel adjunctive treatment for patients with mood and anxiety disorders.

Pre-clinical Studies Linking ω3 PUFAs and Emotional Behavior

To better understand whether the modifications of nutritional ω6/ ω3 PUFA ratio could affect brain function and behavior, studies have been carried out in animals (rodents, monkeys, and pigs) subjected to diets in which PUFAs contents are controlled during one or several generations (de la Presa Owens and Innis, 1999; Clouard et al., 2015). Numerous studies have shown that in

animal models of nutritional ω3 PUFA deprivation, brain DHA levels were decreased while AA levels were increased in several brain areas leading to an imbalance between ω6 and ω3 PUFAs in the ω3 PUFAs deficient mouse brain (Delion et al., 1994; Francès et al., 1995; Favrelière et al., 1998; Carrié et al., 2000b; McNamara and Carlson, 2006; Lafourcade et al., 2011; Larrieu et al., 2012). Nutritional ω3 PUFAs deficiency-induced reduction of brain DHA levels has been associated with the development of depression-like behavior (Carrié et al., 2000a; Takeuchi et al., 2003; DeMar et al., 2006; Fedorova and Salem, 2006; Lafourcade et al., 2011; Larrieu et al., 2012, 2014, 2015; Bondi et al., 2014; Morgese et al., 2016; Manduca et al., 2017). By submitting mice to one generation dietary ω3 PUFAs deficiency, we found that ω3 PUFAs deficient diet alone disturbed social behavior as well as increased anxiety- and depression-related behavior in an open-field and FSTs, respectively (Lafourcade et al., 2011; Larrieu et al., 2012, 2014, 2015). Some studies conducted in rats indicate that the time of immobility in the FST was increased by ω3 PUFAs deficiency (DeMar et al., 2006; Morgese et al., 2016) and reduced by ω3 PUFAs supplementation with fish oil (Naliwaiko et al., 2004; Carlezon et al., 2005; Huang et al., 2008). In addition, the level of DHA in rat whole brains is negatively correlated with the time spent immobile during the FST, a behavioral test used for evaluating the efficacy of compounds rendering or preventing depressive-like states. Interestingly, similar behavioral impairments (e.g., anxiety-like behavior and social interaction) occur in mice after exposure to CSDS, a well-characterized preclinical model of anxiety and depression (Golden et al., 2011; Bosch-Bouju et al., 2016; Larrieu et al., 2017). This model presents strong face validity, as social defeat (e.g., bullying) is a major risk factor to developing depression in humans. One cardinal feature of CSDS is that mice experiencing this chronic stress develop a long-lasting (more than 1 month) aversion to social interaction as well as anhedonia, which can be normalized after chronic (28 days post-CSDS), but not acute administration of antidepressant (Berton et al., 2006; Krishnan et al., 2007) as observed in humans. By comparing the effects of dietary ω3 PUFAs deficiency to those of CSDS on emotional behavior, We found that mice fed with a diet deficient in ω3 PUFAs exhibited behavioral changes and neuronal atrophy profile that resemble those of mice exposed to CSDS (Larrieu et al., 2014). Interestingly, behavioral alterations can be reversed after chronic ω3 PUFAs supplementation. As such, increased anxiety- and depressive-like behavior after chronic stress is normalized after ω3 PUFAs supplementation (Ferraz et al., 2011; Larrieu et al., 2014).

RELEVANT MECHANISMS FOR NUTRITIONAL ω3 PUFA-INDUCED MOOD-RELATED BEHAVIORAL DEFICITS

Numerous epidemiological, clinical, and preclinical studies demonstrated the key role of nutritional ω3 PUFAs in depression and anxiety disorders. In recent years, emphasis was made on identifying molecular and cellular mechanisms by which ω3 PUFAs modulate brain function. ω3 PUFAs and their metabolites are well known to play an important role as signaling molecules that regulate inflammation (Serhan, 2014) and neuroinflammation (recently reviewed in Layé et al., 2018). They also contribute to signal transduction between neurons or neurons and glial cells. Here, we will focus on DHA, the most aggregated fatty acid in the brain while EPA is rapidly b-oxidized and poorly accumulated (Chen and Bazinet, 2015). As DHA is poorly synthesized *de novo*, its brain levels depend on both the dietary supply and blood level bioavailability (Bazinet and Layé, 2014; Lacombe et al., 2018). Once free DHA has entered the brain, it is esterified at membrane phospholipids (both in neurons and glial cells). However, upon neuronal stimulation, injury or stress, DHA is released from phospholipids and can either activate specific receptors or be metabolized into specific derivatives, such as eCBs or oxylipins which regulate specific pathways important to neurotransmission or neuroinflammation (Bazinet and Layé, 2014; Bosch-bouju and Layé, 2016; Layé et al., 2018). In the following section, we first describe the receptors which have been reported to mediate DHA effect in the brain. Then, we focus on the regulation of the eCB system and the HPA axis as recent data show that they could mediate the neuroprotective effect of ω3 PUFAs as both are thought to be involved in depression.

Direct Effect of DHA on Specific Receptors

While free fatty receptors have been widely described to mediate some of the effects of DHA at the periphery, few reports highlight a direct effect of DHA through signaling activity in the brain. In 2000, DHA has been shown to be a ligand of the RXR, the receptor of retinoic acid (a vitamin A metabolite), which heterodimerizes with other nuclear receptors such as retinoic acid receptor, vitamin D receptor, thyroid hormone receptor or PPAR (Lengqvist et al., 2004). DHA effect on neuritogenesis does not involve RXR, as its effect *in vitro* does not activate RXR (Calderon and Kim, 2007). However, DHA potentiates retinoic acid effect and improves cognitive symptoms in a rodent model of Alzheimer disease (Casali et al., 2015) and aged rodents (Létondor et al., 2016). Interestingly, the loss of RXR signaling leads to altered emotional and cognitive behavior in mice (Krzyzosiak et al., 2010; Wietrzych-Schindler et al., 2011). Importantly, DHA antidepressant effect is absent in RXR knock-out mice (Wietrzych-Schindler et al., 2011), further highlighting the role of this receptor and its ligand (possibly DHA and retinoic acid) in emotional behavior. FFAR, members of the "rhodopsin-like" GPCR family, namely GPR40 (FFAR1) and GPR120 (FFAR4), have been recently highlighted as potentially mediating LC FFAs signal from pancreatic beta-cells as well as the intestines (Itoh et al., 2003; Hirasawa et al., 2005). These lipid receptors were also reported to be present in the brain (Ma et al., 2007; Dragano et al., 2017). Memory-induced progenitor cell proliferation and DHA-induced neurogenesis in the hypothalamus are mediated by GPR40 (Ma et al., 2008; Yamashima, 2008; Nascimento et al., 2016). In addition, DHA-induced GPR40 signaling pathway activates β-endorphin release in the hypothalamus of rodents

(Nakamoto et al., 2015). Importantly, the chronic activation of GPR40 signaling in the brain reduces depressive-like behavior (Nishinaka et al., 2014). In addition, anxiety-like behavior and sucrose preference, a behavioral sign of anhedonia, are reduced in GPR40 knock-out mice further highlighting the role of GPR40 signaling in the pathophysiology of mood disorders (Aizawa et al., 2016). GPR120, another GPR which signals DHA activity, is highly expressed in the arcuate nucleus of the hypothalamus and the NAc, a structure involved in emotional behavior (Auguste et al., 2016). Interestingly, GPR120 activation by a specific agonist reduces obesity-induced emotional behavior alteration (Auguste et al., 2016). Taken together, these data suggest that several receptors could mediate a direct effect of DHA on neurons to control emotional behavior, opening new avenues in drug development targeting these receptors. However, additional studies are needed to determine whether DHA acts through these receptors to protect from depression and anxiety disorders in humans.

Endocannabinoid System

Regulation of the eCB system could mediate the neuroprotective effect of ω3 PUFAs as both are thought to be involved in depression. The eCB system is in a unique position to link food lipids, neuroplasticity and behavior (Bazinet and Layé, 2014; Bosch-bouju and Layé, 2016; Chianese et al., 2017). eCBs are signaling lipids produced from membrane LC fatty acid in response to neuronal activity and they bind the GPCR CB1R (Mackie, 2008) (**Figure 2**). eCBs are produced on-demand and are rapidly degraded, back into PUFAs or oxidized into active metabolites (Bosch-bouju and Layé, 2016). eCBs include the fatty acid AEA, DHEA, oleylethanolamide and palmitoylethanolamide, as well as 2-AG (Piomelli and Sasso, 2014). The two principal eCBs, AEA and 2-AG, are AA-derived metabolites, while DHEA is derived from the DHA and oleylethanolamide and palmitoylethanolamide is derived from EPA. The most well-studied eCBs are the ω6 PUFA-derived AEA (Devane et al., 1992) and the 2-AG (Sugiura et al., 1995) as compared to the ω3 PUFA-derived eCBs. Activation of CB1 receptors inhibits AC activity leading to a subsequent reduction in the cAMP cascade, augmentation of potassium channels, and inhibition of subsequent calcium influx via calcium channels (**Figure 2**) (Howlett and Fleming, 1984; Howlett, 2002). Consequently, the activation of the CB1R inhibits the release of both excitatory (glutamate) and inhibitory GABA neurotransmitters from presynaptic neurons (Wilson and Nicoll, 2001, 2002; Freund et al., 2003) (**Figure 2**). Finally, numerous important studies have unraveled the key role of eCB system in mood regulation (Morena et al., 2015; Hill and Lee, 2016). As eCBs are derived from ω6 and ω3 PUFA precursors, we hypothesized that the effects of PUFAs on mood-related behavior might be mediated, at least partly, through the eCB system (Lafourcade et al., 2011; Bosch-Bouju et al., 2016; Manduca et al., 2017). As such, inadequate PUFAs ratio during critical time window, i.e., gestation or lactation can lead to changes in eCBs contents in the brain. Newborn piglets that were fed with a diet containing ALA, AA and DHA during the first 18 days of life showed an expected increase of AA and DHA levels in the brain but also of AEA and DHEA metabolites (Berger et al., 2001). Watanabe et al. reported that nutritional ω3 PUFAs deficiency for 2 generations elevates the levels of 2-AG in the mouse brain while ω3 PUFAs supplementation reduces them. In this study, DHA brain levels were affected by dietary ω3 PUFAs deficiency, but not AA the precursor of 2-AG which remained unchanged as compared to the control diet group (Watanabe et al., 2003). It is now well documented that AA levels are barely impacted by PUFAs content of the diet while DHA brain levels are more sensitive to dietary ω6/ω3 PUFAs. Whether increased 2-AG and AEA after exposure to a diet rich in ω6 PUFAs is a compensatory effect to buffer AA concentrations remains to be determined. Lastly a 2-week-supplementation in DHA increased the DHEA and decreased the AEA in brain homogenates in both rats and mice (Wood et al., 2010). One *in vitro* study also demonstrated that unesterified free DHA could directly regulate CB1 gene expression in hippocampal neurons (Pan et al., 2011). Collectively, these reports support the hypothesis proposing that nutritional PUFAs intake is tightly linked to brain eCB levels. By regulating levels of eCBs in the brain, PUFAs have been shown to impact hippocampal synaptic plasticity (Thomazeau et al., 2017) and eCB-dependent plasticity (Lafourcade et al., 2011; Manduca et al., 2017) as well as CB1-associated signaling pathways (Larrieu et al., 2012) in the PFC and NAc. In mice, perinatal exposure to dietary ω3 PUFA deficiency, which leads to low DHA levels in the PFC and the NAc, abolished the eCB-long-term depression in these brain structures. Specifically, this alteration is mediated by an uncoupling from CB1R to its G protein (Lafourcade et al., 2011). Moreover, the effect of the CB1 agonist WIN55,212-2 in anxiety-like behavior was abolished and the CB receptor signaling pathways were altered in the PFC and hypothalamus of ω3 PUFA-deficient mice (Larrieu et al., 2012). A recent study has involved the 2-AG in these aforementioned alterations. Our recent work highlighted that the inhibition of 2-AG degradation normalized emotional behavior deficits and eCB-dependent synaptic plasticity alteration observed in ω3 PUFA-deficient adult mice (Manduca et al., 2017). These observations are the first synaptic and molecular evidence that malnutrition related to ω6/ ω3 PUFAs ratio can have detrimental effect on eCB system, subsequently leading to impaired behavior.

Hypothalamic–Pituitary–Adrenal Axis

Stress and high trait anxiety are a major risk factor for neuropsychiatric diseases, particularly major depression and anxiety disorders, and are etiologically causal in PTSD (Sandi and Richter-Levin, 2009). Interestingly, although several mechanisms underlying the effects of dietary ω3 PUFA deficiency on emotional behavior have been described (e.g., eCB system), those specifically related to HPA axis function remain poorly understood. Nevertheless, clinical data reported that low plasma DHA levels correlate with higher cerebrospinal fluid CRH levels (Hibbeln et al., 2004) and with higher cortisol in plasma (Nieminen et al., 2006; Mocking et al., 2013). Healthy men receiving supplementation for 3 weeks with dietary fish oil display a blunted cortisol response after an acute mental stress (Delarue et al., 2003). Mood-related deficits observed in

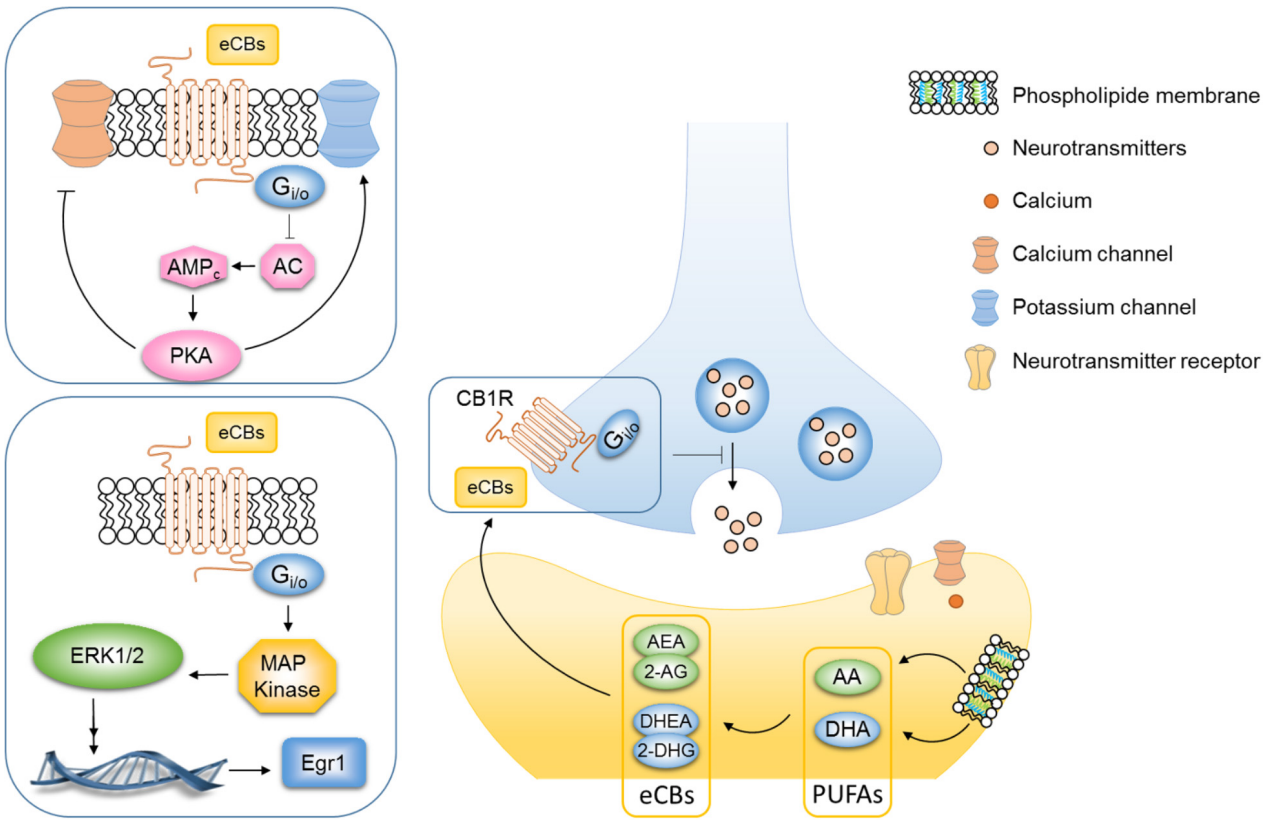

FIGURE 2 | PUFAs are key actors in the regulation of endocannabinoid system. Endocannabinoids are signaling lipids produced from membrane long-chain fatty acid in response to neuronal activity that bind the G-protein-coupled receptor (GPCR) CB1R. The two principal eCBs, AEA and 2-AG, are AA-derived metabolites while DHEA derived from the DHA. eCBs are released into the synaptic cleft and then bind the CB1R on the presynaptic neuron. Activation of CB1R inhibits adenylyl cyclase (AC) activity leading to a subsequent reduction in the cyclic adenosine monophosphate (cAMP) cascade, augmentation of potassium channels, and inhibition of subsequent calcium influx via calcium channels. Consequently, the activation of the CB1R inhibits the release of both excitatory (glutamate) and inhibitory (GABA) neurotransmitters from the presynaptic neuron and decreases synaptic plasticity. The stimulation of CB1R by CB agonists (THC, WIN55,212-2, and CP-55940) or eCBs also activate MAPK signaling pathway. Both eCB-dependent plasticity and CB1R-dependent signaling pathway in brain areas involved in mood-regulation are altered in mice that chronically fed an omega-3 deficient diet.

deficient mice or rats were recently linked to disrupted GR-mediated signaling pathway, HPA axis hyperactivity as well as eCB system impairment, all involved in mood regulation (Ferraz et al., 2011; Lafourcade et al., 2011; Larrieu et al., 2014, 2015; Bosch-Bouju et al., 2016). Rats that were fed with a ω3 PUFA deficient diet display HPA axis hyper-reactivity after stress exposure reflected by increased levels of plasma corticosterone compared to control diet group (Levant et al., 2008). Conversely, corticosterone hypersecretion induced by a chronic stress and IL-1β exposure is dampened in ω3 PUFA supplemented rats (Song et al., 2003; Ferraz et al., 2011). In Morgese et al. (2016), increased hypothalamic CRF release as well as increased plasmatic corticosterone levels has been shown in ω3 PUFA deficient rats, further demonstrating the link between HPA axis hyperactivity and dietary ω3 PUFAs. In a recent study, we demonstrated that anxiety- and depressive-related behaviors as well as neuronal atrophy in the medial PFC observed in mice fed with a diet deficient in ω3 PUFAs are both mediated by HPA axis hyperactivity (Larrieu et al., 2014). ω3 PUFAs supplementation beyond weaning prevents chronic stress-induced increases in plasma corticosterone levels (Ferraz et al., 2011; Larrieu et al., 2014; Meneses et al., 2017), PFC neuronal shrinkage (Larrieu et al., 2014) as well as anxiety- and depressive-like behaviors (Ferraz et al., 2011; Larrieu et al., 2014). In another study, we confirmed and followed up on their initial observations by demonstrating that GR signaling pathway is compromised in the PFC of ω3 PUFA-deficient mice along with dendritic arborization atrophy (Larrieu et al., 2015). The modulation of neuronal morphology by ω3 PUFAs might be not a generalized phenomenon since neuronal arborization atrophy is only observable in the PFC but not the CA1 of the hippocampus of ω3 PUFA-deficient mice (Delpech et al., 2015b; Larrieu et al., 2015). To further establish the link between dietary ω3 PUFAs consumption and neuronal morphology, *in vitro* studies were conducted showing that PUFAs activate neurites formation and growth in hippocampal (Calderon and Kim, 2004; Cao et al., 2009) and cortical neurons (Cao et al., 2005) in primary culture. Moreover, in these same cultures, a decrease of DHA leads to the reduction of the size of neurites (Ikemoto et al., 1997; Furuya et al., 2002; Calderon and Kim, 2004; Cao et al., 2009).

The accretion of DHA in the brain considerably facilitates the formation of the dendritic spines in the hippocampus of Gerbils that were fed with a diet supplemented in DHA (Sakamoto et al., 2007). Interestingly, genetically modified Fat-1 mice that are able to catalyze the conversion of ω6 into ω3 PUFAs display a higher density of spines in the hippocampus compared to WT mice (Kang et al., 2004; He et al., 2009). Moreover, increased spine density in the hippocampus of Fat-1 mice is associated with better cognitive performances assessed in Morris water maze along with increased adult neurogenesis (He et al., 2009). The protective effect of LC ω3 PUFAs could be linked to hippocampal neurogenesis (recently reviewed in (Zainuddin and Thuret, 2012). As such, changes in hippocampal neurogenesis and cell survival in the dentate gyrus have been correlated with depressive-like behavior. A recent study demonstrated that clamping glucocorticoid levels prevent CSDS-induced decreases in neurogenesis and depressive-like behavior in wild type mice, but not in mice with a genetic ablation of neurogenesis (Lehmann et al., 2013). This is particularly relevant knowing that LC ω3 PUFAs supplementation prevents CSDS-induced HPA axis dysregulation (Larrieu et al., 2014). However, whether the beneficial effect of ω3 PUFAs on glucocorticoids and mood is dependent on neurogenesis remains to be evaluated. Finally, an elegant study showed that EPA but not DHA increases neural stem cell proliferation reflected by an increased number of neurospheres bulk via CB1R activity (Dyall et al., 2016). The findings that ω3 PUFAs alone modulate neuronal arborization as well as adult neurogenesis highlight the role of PUFAs as a potent modulator of brain health. Taken together, these studies provide strong validity of nutritional ω3 PUFA-deficient diet as one of the many faces of stress that deeply affects GR-dependent HPA axis function and neuronal morphology plasticity in brain areas associated with emotional behavior.

Neuroinflammatory Pathways

Inflammation is a key mechanism in the pathophysiology of mood disorders, including major depression, post-partum depression and bipolar disorder (Dantzer et al., 2008; Capuron and Miller, 2011). Increased levels of inflammatory factors, such as proinflammatory cytokines and chemokines, are found in a subset of depressed patients and may contribute to their symptoms through a direct effect in the brain (Raison and Miller, 2011). The mechanisms underlying inflammation and depression have been thoroughly reviewed elsewhere (Capuron and Castanon, 2017). Enhanced peripheral inflammation has also been reported in PTSD (Gill et al., 2008; Newton et al., 2014; Passos et al., 2015; Lerman et al., 2016) and bipolar disorder (Goldstein et al., 2009; Fiedorowicz et al., 2015; Kalelioglu et al., 2015; Uyanik et al., 2015). Importantly, inflammation has been proposed to be key in stress vulnerability and the pathogenesis of major depression (Ménard et al., 2017).

Long chain ω3 PUFAs, DHA, and EPA and their derivatives, so-called SPMs, are well-known regulators of the inflammatory response (Serhan, 2014, 2017). More recently, DHA, EPA, and their derivatives have been shown to also regulate neuroinflammatory processes (Kavanagh et al., 2004; Li et al., 2015; Rey et al., 2016; Dong et al., 2017; Fourrier et al., 2017; Shi et al., 2017; recently reviewed in Layé et al., 2018). Briefly, the expression of the pro-inflammatory cytokine TNFα, IL-6, and IL-1β in the brain (triggered by peripheral or intracerebral administration of LPS, the Gram-negative bacteria endotoxin, amyloid beta administration or associated to aging) is decreased by DHA and EPA dietary supplementation (Labrousse et al., 2012; Orr et al., 2013; Dehkordi et al., 2015; Hopperton et al., 2016). Importantly, in regards to the protective effect of EPA in depression, a dietary supplementation with this fatty acid decreased TNFα expression in the hippocampus following IL-1β central injection (Dong et al., 2017). *In vitro*, DHA, and EPA directly target microglia, the brain innate immune cell (De Smedt-Peyrusse et al., 2008; Antonietta Ajmone-Cat et al., 2012; Pettit et al., 2013; Chang et al., 2015; Fourrier et al., 2017), however, a direct effect of these fatty acids on microglia *in vivo* has not been studied yet. In a model of multiple sclerosis induced by cuprizone, DHA/EPA promote the shift of microglia polarization toward a repair non-inflammatory phenotype (Chen et al., 2014). We have found that the brain content of ω3 PUFA, either increased through the diet or by genetic means, influences microglia and the related neuroinflammatory response to LPS (Mingam et al., 2008; Madore et al., 2014; Delpech et al., 2015a,b; Dinel et al., 2016). In rodent models of neuroinflammation triggered by the intracerebral administration of amyloid-β or cuprizone, brain DHA decreases the number of activated microglia, but not of astrocytes (Hopperton et al., 2016), and promotes an anti-inflammatory phenotype of microglia (Chen et al., 2014). An acute intravenous administration of DHA reduces LPS-induced cytokine production in the hippocampus (Fourrier et al., 2017), but no significant effect of intravenously administered DHA was shown on microglia activation (measured by the upregulation of translocator protein TSPO by Positron-emission tomography) in the injured spinal chord of rat (Tremoleda et al., 2016). DHA and EPA effect on neuroinflammatory pathways could be either direct or indirect. Indeed, LC-PUFAs are converted by COX, LOX, and CYP450 into SPMs, which display pro or anti-inflammatory activities (Chiang and Serhan, 2017), including in the brain (Orr et al., 2013; Rey et al., 2016; Layé et al., 2018). Eicosanoids, resolvins, protectin and maresin derived from DHA and EPA have anti-inflammatory and pro-resolving activities (Bazan, 2009; Serhan et al., 2011). On the opposite, SPMs derived from LA and AA (prostaglandins, leukotrienes or thromboxanes) are mostly pro-inflammatory (Calder, 2006). *In vitro*, DHA derivatives display anti-inflammatory activities in microglia (Marcheselli et al., 2003; Lukiw et al., 2005; Orr et al., 2013; Rey et al., 2016). Brain inflammation triggered by the administration of LPS activates ω6 PUFA derived-prostaglandins production in the brain (Rosenberger et al., 2004; Taha et al., 2017), together with the expression of the enzymes involved in the synthesis of SPMs (Rosenberger et al., 2004; Taha et al., 2017). However, recent work showed that amyloid-β brain infusion, which is proinflammatory, did not increase brain SPMs production (Hopperton et al., 2018). Importantly, PUFAs dietary intervention can modulate cellular levels of both PUFAs and SPMs, with dietary ω6 PUFAs supplementation increasing AA-derived and decreasing EPA-derived SPMs (Taha et al., 2016).

Conversely, LC ω3 PUFAs supplementation increasing EPA and DHA-derived SPMs (Balvers et al., 2012; Hashimoto et al., 2015) have not been consistently demonstrated (Hopperton et al., 2018). These observations reinforce the need for more studies to link nutritionnal interventions and SPMs production in specific brain regions.

As previously described, clinical trials using DHA and/or EPA showed mixed results on depressive symptoms. However, based on meta-analysis, EPA has been suggested as a predictor of mood disorder treatment efficiency (Martins, 2009; Sublette et al., 2011; Mocking et al., 2016). Such a positive effect of EPA could be linked to its anti-inflammatory activity. Indeed, in depressed patients, high EPA supplementation is more effective in those with inflammation (Rapaport et al., 2016). In particular, patients with high IL-1 receptor antagonist and C-reactive protein blood levels have greater improvement in mood symptoms in response to EPA, but not DHA enriched dietary supplement. Additional studies with a higher number of patients are warranted to confirm this interesting first study. In addition, whether the higher efficiency of high EPA rather of DHA dietary supplementation is linked to its specific effect on inflammation through specific SPMs remains to be investigated.

CONCLUSION AND FUTURE DIRECTIONS

As indicated above, the summarized literature indicate that low ω3 PUFAs intake may predispose certain individuals to depression and anxiety and that dietary supplementation with LC ω3 PUFAs represents an interesting strategy for preventing or treating depression and anxiety disorders in certain individuals. However, several important issues remain to be determined. One of those is the discordant results regarding outcomes in clinical nutritional interventions to investigate the effectiveness of ω3 PUFA supplementation on mood. The unmatched results seem to be partly due to the lack of standardization regarding important parameters such as (i) the inclusion criteria used, (ii) the PUFA composition of the fish oil as well as (iii) the nutritional baseline status of subjects, and (iv) the methods of diagnosis used. We are now beginning to understand how PUFAs affect our brain through a direct sensing effect or an indirect one. This review highlights that ω3 PUFAs, in particular DHA, act onto the brain through a direct effect on FFAR or other indirect mechanisms. We also discussed an indirect effect of ω3 PUFAs on eCB and the HPA axis systems as relevant mechanisms by which dietary ω3 PUFAs modulate mood-related behaviors. Although recent work suggest a causal relationship between nutritional ω3 PUFAs deficiency and alterations of these two systems, major questions remain unanswered, such as how dietary ω3 PUFA maintains HPA axis function to prevent emotional impairment. In this review, we highlighted how powerful dietary PUFAs are in the modulation of the eCB system, which is known to be intimately involved in the regulation of the HPA axis (McLaughlin et al., 2014). As to whether these two mechanisms are interconnected in the effects of ω3 PUFA deficiency-induced depression is yet to be determined. In conclusion, this review reinforces the idea of the usefulness of the dietary ω3 PUFAs as an interesting tool for the design and testing of new non-pharmacological strategies in the treatment of neuropsychiatric disorders such as mood-related disease.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Brain Ceramide Metabolism in the Control of Energy Balance

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The regulation of energy balance by the central nervous system (CNS) is a key actor of energy homeostasis in mammals, and deregulations of the fine mechanisms of nutrient sensing in the brain could lead to several metabolic diseases such as obesity and type 2 diabetes (T2D). Indeed, while neuronal activity primarily relies on glucose (lactate, pyruvate), the brain expresses at high level enzymes responsible for the transport, utilization and storage of lipids. It has been demonstrated that discrete neuronal networks in the hypothalamus have the ability to detect variation of circulating long chain fatty acids (FA) to regulate food intake and peripheral glucose metabolism. During a chronic lipid excess situation, this physiological lipid sensing is impaired contributing to type 2 diabetes in predisposed subjects. Recently, different studies suggested that ceramides levels could be involved in the regulation of energy balance in both hypothalamic and extra-hypothalamic areas. Moreover, under lipotoxic conditions, these ceramides could play a role in the dysregulation of glucose homeostasis. In this review we aimed at describing the potential role of ceramides metabolism in the brain in the physiological and pathophysiological control of energy balance.

Keywords: hypothalamus, lipid sensing, lipotoxicity, ceramides, energy homeostasis

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HYPOTHALAMIC LIPID METABOLISM: A BASIC PATHWAY REGULATING ENERGY BALANCE

The hypothalamus regulates a vast number of homeostatic functions. Among them, regulation of endocrine axes, reproductive function, and energy balance are of particular importance (Williams et al., 2001; King, 2006). Despite the well-established role of neuropeptides, several lines of evidence have demonstrated that modulation of hypothalamic lipid metabolism is a very important mechanism regulating energy balance. Indeed, while neuronal activity primarily relies on glucose, the brain expresses at high level enzymes responsible for the transport, utilization and storage of lipids. Since the work of Oomura et al. (1975), growing body of evidence suggests that fatty acids (FA) are able to modulate neuron activity in hypothalamus and regulate energy balance through the control of insulin secretion, hepatic glucose production, adipose storage and food intake (Obici et al., 2002; Cruciani-Guglielmacci et al., 2004; Lam et al., 2005). This phenomenon has been called "lipid sensing," and the molecular mechanisms involved are still matter of controversy. It includes plasma membrane proteins such as G-protein coupled receptor 120 (GPR120) or FA translocase (FAT/CD36), but also intracellular events including FA oxidation or synthesis of diacyl-glycerol (DAG) and ceramides (Magnan et al., 2015). In addition lipid membrane composition itself may regulate neuronal signaling pathways as the lipid profile in specific microdomains named lipid rafts

(enriched in cholesterol, saturated phospholipids and sphingolipids) could modulate the signaling pathway integration through changes in the affinity of proteins to concentrate in these domains (Yaqoob and Shaikh, 2010). Interestingly, key enzymes involved in FA synthesis and oxidation, namely acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), malonyl-CoA decarboxylase (MCD) and carnitine palmitoyltransferase 1 (CPT1) are expressed at high levels in the arcuate (ARC), paraventricular (PVH), dorsomedial (DMH), and ventromedial (VMH) nuclei, which are, with the lateral hypothalamic area, among the most relevant hypothalamic sites modulating energy homeostasis (Dowell et al., 2005; Lopez et al., 2007; Gautron et al., 2015). AMP-activated protein kinase (AMPK), a cellular energy sensor that modulates FA metabolism by controlling ACC and MCD activities and FAS expression, is also highly expressed in the hypothalamus (Lage et al., 2008; Carling et al., 2011; Hardie et al., 2012; Lopez et al., 2016).

In addition to this anatomical data, physiological, pharmacological and genetic evidence has shown that the modulation of these activities at hypothalamic level impacts energy homeostasis. Thus, treatments with FAS inhibitors, such as cerulenin and C75 (Loftus et al., 2000; Hu et al., 2003), and with factors that decrease FAS expression, such as leptin, tamoxifen, and estradiol (Lopez et al., 2006; Wolfgang et al., 2007; Martinez de Morentin et al., 2015), as well as the specific ablation of hypothalamic FAS (Chakravarthy et al., 2007) induce a remarkable weight loss and hypophagic effect, which depends on accumulation of malonyl-CoA (the product of ACC and the substrate of FAS) in the hypothalamus. Of note, this anorectic action is linked to decreased expression of orexigenic (AgRP and NPY) neuropeptides and elevated expression of anorexigenic (CART, POMC) ones in the ARC (Loftus et al., 2000; Hu et al., 2003; Lopez et al., 2006; Chakravarthy et al., 2007; Wolfgang et al., 2007). One interesting possibility to explain this action is the inhibitory effect of malonyl-CoA on CPT-1a, therefore preventing the access of long-chain fatty acyl-CoAs to the mitochondria and leading to its cytoplasmic accumulation which would be sensed as a signal of nutrient abundance. This idea is supported by the fact that genetic ablation of hypothalamic CPT-1a activity reduces food intake (Obici et al., 2003; Wolfgang et al., 2006, 2008).

Hypothalamic AMPK plays a major role in the modulation of both feeding (Andersson et al., 2004; Minokoshi et al., 2004; Claret et al., 2007; Andrews et al., 2008; Lopez et al., 2008, 2016) and energy expenditure, specifically through the control of hormone-induced brown adipose tissue (BAT) thermogenesis. Specifically, within the VMH, a decreased AMPK activity activates BAT thermogenesis through increased sympathetic nervous system (SNS) outflow. Notably, this pathway, initially described for central effects of thyroid hormones on energy balance (Lopez et al., 2010), is also shared by leptin (Tanida et al., 2013), BMP8B (bone morphogenetic protein 8B) (Whittle et al., 2012; Martins et al., 2016), estrogens (Martinez de Morentin et al., 2014, 2015), glucagon-like-peptide 1 agonist (Beiroa et al., 2014) and nicotine (Martinez de Morentin et al., 2012; Seoane-Collazo et al., 2014). Finally, we proposed the VMH AMPK-SNS-BAT axis as a canonical mechanism modulating energy homeostasis (Lopez et al., 2013, 2016; Contreras et al., 2015).

HYPOTHALAMIC LIPOTOXICITY: A PATHOPHYSIOLOGICAL MECHANISM OF OBESITY

In peripheral tissues, accumulation of reactive lipid species, such as DAG, free fatty acids, free cholesterol, and ceramides is a pathogenic mechanism of insulin resistance, type 2 diabetes, liver and cardiovascular disease (Chaurasia and Summers, 2015). This lipotoxicity occurs through inflammation and endoplasmic reticulum (ER) stress (Ozcan et al., 2004; Martinez de Morentin and Lopez, 2010; Unger et al., 2010; Virtue and Vidal-Puig, 2010; Bellini et al., 2015), which, of note, can also occur in the central nervous system (CNS), as observed in certain neurodegenerative disorders (i.e., polyglutamine diseases, Parkinson's disease and amyotrophic lateral sclerosis) (Ilieva et al., 2007). In particular previous studies have demonstrated that ER stress and activation of the unfolded protein response played a key role in promoting insulin resistance in peripheral tissues (Kammoun et al., 2009). In the hypothalamus, ER stress also induces insulin resistance, and leptin resistance, leading to weight gain (Zhang et al., 2008; Ozcan et al., 2009). Moreover, a chronic lipid excess condition, such as overweight and obesity, has been shown to impair lipid sensing, and this deregulation—namely brain lipotoxicity—may contribute to the setting of type 2 diabetes in predisposed subjects through changes in autonomic nervous system activity (Picard et al., 2014a). However, one key question that remains to be addressed relates to the status of lipid metabolism and whether accumulation of specific lipid species occurs in the hypothalamus. Recent studies point out that ceramides accumulation under lipotoxic conditions could play a role on the deregulation of energy balance in both hypothalamic and extra-hypothalamic areas (Le Stunff et al., 2013; Contreras et al., 2014; Picard et al., 2014b).

DE NOVO CERAMIDE BIOSYNTHESIS IN BRAIN

In peripheral organs, ceramides are important mediators of lipotoxicity: they accumulate in insulin-sensitive tissues and in pancreatic β cells during the development of obesity, and their intracellular levels correlate with both insulin resistance and β cell apoptosis (Bellini et al., 2015). In rodents, it has been demonstrated that enzymes of *de novo* ceramides synthesis are expressed in hypothalamus and hippocampus (Contreras et al., 2014; Picard et al., 2014b).

In the context of obesity-associated lipid excess, *de novo* ceramides are mainly produced from saturated FA such as palmitate, and this synthesis begins in the cytoplasmic face of the ER (**Figure 1**). The first step is the condensation of L-serine with palmitoyl-CoA to form 3-ketosphinganine, catalyzed by serine palmitoyl-transferase (SPT) (Hannun and Obeid, 2008). Then 3-ketosphinganine is reduced to dihydrosphingosine (DH-Sph) by 3-ketosphinganine reductase and the resulting

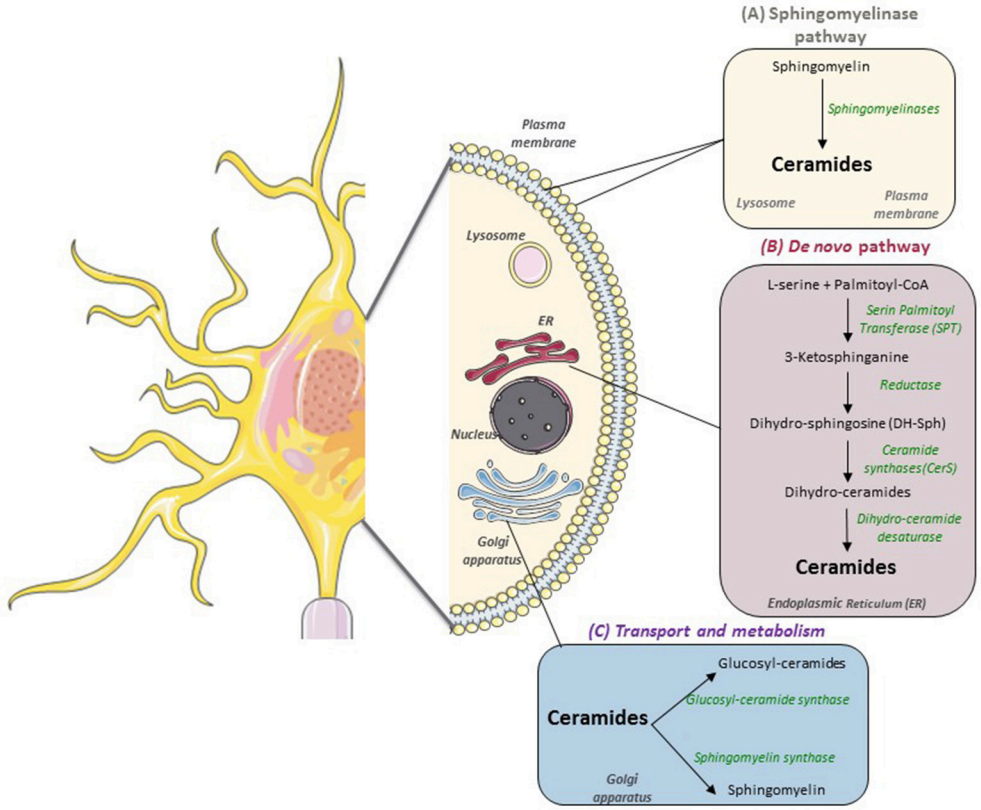

FIGURE 1 | Sphingolipids metabolism in nervous cells. In mammals, there are two main pathways to produce sphingolipids: **(A)** the catabolic sphingomylinase pathway that takes place in the lysosomal and plasma membranes and leads to the degradation of sphingomyelin (SM) into ceramides by Sphingomyelinases (SM); **(B)** the *de novo* synthesis pathway which starts on the cytoplasmic face of the endoplasmic reticulum (ER) with the condensation of Palmitoyl-CoA and L-Serine to form 3-ketosphinganine. **(C)** Then, ceramides are transported to the Golgi apparatus to be metabolized into more complex sphingolipids such as glucosyl-ceramides and sphingomyelin.

DH-Sph acts as a substrate for ceramide synthases (CerS), leading to the production of dihydroceramides. In mammals, six CerS isoforms are expressed, they have distinct specificities depending on the acyl-CoA chain length they use for N-acylation of DH-Sph (Pewzner-Jung et al., 2006; Mullen et al., 2012) Dihydro-ceramides are transformed into ceramides by the dihydroceramide desaturase DES1 (Causeret et al., 2000). Ceramides are then transported to the Golgi apparatus where they are converted into sphingomyelin or into glucosyl-ceramides by sphingomyelin synthase and glucosyl-ceramide synthase, respectively (Hanada et al., 2003).

Glucosylceramide synthase (GCS) derived gangliosides are acidic glycosphingolipids that are prominently expressed by neurons (Jennemann et al., 2005). They contribute to the formation of membrane microdomains which regulate intracellular signal transduction (Simons and Gerl, 2010). In particular, Nordström et al. have recently demonstrated that adequate function of the hypothalamic leptin receptor (ObR) requires GCS expression (Nordstrom et al., 2013).

In addition to *de novo* synthesis pathway, degradation of sphingomyelin into ceramide by sphingomyelinases is another metabolic pathway which leads to ceramide production, it takes place in the lysosomal membrane and in the cytoplasmic membrane (Hannun and Obeid, 2008). Of note, a mutation in Sphingomyelin phosphodiesterase 1 (also known as acid sphingomyelinase, ASM) causes Niemann-pick disease, characterized by the buildup of toxic amount of sphingomyelin and leading to multi-organ dysfunction (including profound brain damage) (Schuchman and Desnick, 2017).

CERAMIDES AND BRAIN LIPOTOXICITY

It has been shown that exogenous ceramides could induce hypothalamic lipotoxicity, ER stress and decreased sympathetic tone to the BAT, which leads to decreased thermogenesis and feeding-independent weight gain (Contreras et al., 2014). In addition, genetic modulation of ceramide-induced ER stress pathway in the VMH modulates energy balance by influencing BAT thermogenesis and insulin sensitivity, as well as promoting an overall improvement of the metabolic phenotype of leptin and insulin resistant obese rats (Contreras et al., 2014). In this work, genetic overexpression of GRP78 (the chaperone glucose-regulated protein 78) in the VMH of rats abolishes ceramide

action by reducing hypothalamic ER stress and increasing BAT thermogenesis, which lead to weight loss and improved glucose homeostasis. Overall, these data identify a signaling network involving central ceramides, hypothalamic lipotoxicity/ ER stress and BAT thermogenesis as a pathophysiological mechanism of obesity. In addition, the amelioration of ER stress by overexpression of GRP78 does no impact ceramide levels in obese Zucker rats, which remain elevated when compared with their lean littermates (Contreras et al., 2017). Therefore, this evidence indicates that ER stress is downstream ceramide's effect (Contreras et al., 2017).

Interestingly, ER stress *per se* could also lead to an increased ceramide synthesis. It has been shown in rodents that ER stress is concomitant with liver insulin resistance and is able to activate SREBP-1c cleavage (Kammoun et al., 2009), and to induce the whole hepatic lipogenic program, thus leading to steatosis and increased ceramide content (Holland and Summers, 2008). Whether a similar mechanism operates in the brain is currently unknown. In addition, it has been shown in peripheral organs that, depending on the ceramide chain length and saturation, the effects could be very different. For example, CerS 1 is mainly involved in the synthesis of C18:0 ceramides, and it has been linked to a greater insulin sensibility in muscle cells, conversely to other CerS isoforms (Frangioudakis et al., 2013). In brain, Zhao et al. reported that Cers1 deficiency dramatically affects sphingolipid homeostasis and leads to Purkinje cell loss, lipofuscin accumulation and overall functional deficit in mice (Zhao et al., 2011; Ginkel et al., 2012).

A SPECIFIC ROLE FOR CPT-1C IN BRAIN CERAMIDE METABOLISM?

Recently, the brain specific isoform of carnitine palmitoyl-transferase, CTP-1c, has been involved in ceramide metabolism and suggested to be a potential downstream effector of leptin action on the control of feeding (Gao et al., 2011). As it has been already demonstrated, leptin inhibits AMPK in the ARC, thus leading to ACC activation and increased malonyl-coA levels (Minokoshi et al., 2004). Gao et al. suggested that CPT-1c, located in the ER, could be a downstream target in the mediation of malonyl-CoA's anorectic signaling action: malonyl-CoA could inhibit CPT-1 to reduce ceramide *de novo* biosynthesis, or it could interact with another target to decrease ceramide level (Gao et al., 2011). Fine molecular studies demonstrated that CPT-1c had a very weak acyl-transferase activity (20–300 times less than CPT-1a and−1b) and preferentially used palmitoyl-CoA as substrate (Sierra et al., 2008). In addition, a significant portion of CPT-1c is localized in the ER. Taken together these data lead to the hypothesis that CPT-1c is involved in ceramide metabolism. Consistently, Gao et al. demonstrated that CPT-1c overexpression in ARC lead to increased ceramide levels whereas the CPT-1c deletion had the opposite effect, and that ceramide metabolism in the Arc was required for leptin's anorectic actions (Gao et al., 2011).

Recent evidence shows that ghrelin (a stomach-derived orexigenic hormone) induces hypothalamic AMPK activation, which decreases ACC activity, reducing malonyl-CoA concentration and therefore releasing inhibition of CPT-1c (Ramirez et al., 2013). CPT1c activity—as explained before—promotes elevated ceramide synthesis and accumulation, which elicits *agrp* and *npy* gene expression and subsequently hyperphagia. Interestingly central inhibition of ceramide synthesis with myriocin negates the orexigenic action of ghrelin through the normalization of orexigenic neuropeptide levels, pointing out a direct role for hypothalamic ceramides in the control of food intake (Ramirez et al., 2013). The authors further demonstrate that CPT-1c is required to mediate the anorectic action of leptin in mice, and that both CPT-1c and ceramide downregulation in hypothalamus are specifically required for the malonyl-coA anorectic action (Gao et al., 2011).

LIPID METABOLISM IN OTHER BRAIN REGIONS CONTRIBUTES TO THE REGULATION OF ENERGY HOMEOSTASIS

Besides the hypothalamus, other brain areas have been shown to be involved in the regulation of energy homeostasis. Regarding food behavior, satiation signals arising in the gastro-intestinal (GI) system converge on the dorsal hindbrain and are integrated with taste and other inputs (Schwartz et al., 2000; Woods, 2009). The dorsal hindbrain connects directly with the ventral hindbrain, where neural circuits direct the autonomic nervous system to influence blood glucose, and where the motor control over eating behavior is located (Woods and D'Alessio, 2008). The hypothalamus and other brain areas, such as hippocampus and striatum, integrate satiation, adiposity and nutrient signals with time of day and other factors like experience, social situation, and stressors. Once integrated, output signals regulate feeding behavior (including food preference, hedonic behavior), motivation (to search food), learning as well as energy expenditure or glucose homeostasis (Woods and D'Alessio, 2008; Woods, 2009). The hippocampus itself is described as a regulator of feeding behavior and body weight regulation (Davidson et al., 2007). Recently, Picard et al. demonstrated that a decreased TG-hydrolysis in hippocampus, through pharmacological or genetic inactivation of lipoprotein lipase (LPL), lead to obesity in both rats and mice (Picard et al., 2014b). In addition, data shows that obesity-associated cognitive impairment could be improved by selectively lowering TG, while intracerebroventricular (ICV) injection of triolein impairs learning in normal mice (Farr et al., 2008). Taken together, these observations raise the possibility that nutritional lipids, and particularly TG, could directly affect the encoding of reward in the mesocorticolimbic system (Farr et al., 2008). Indeed, TG processing enzymes and lipoprotein receptors are expressed in the brain, and several lines of evidence indicate that circulating TG-rich particles access the brain (Wang and Eckel, 2012).

The intra-hippocampal LPL inhibition leads to increased body weight due to decreased locomotor activity and energy expenditure but with no change in food intake, concomitant with high parasympathetic tone (Picard et al., 2014b). Interestingly, Magnan and colleagues identified *de novo*

TABLE 1 | Summary of the main effects and mediators of central ceramide actions.

Area of the brain	Ceramide modulation	Consequences	References
Hippocampus	LPL inhibition increases *de novo* ceramide biosynthesis.	Increased body weight gain, decreased locomotor activity, high parasympathetic tone.	Picard et al., 2014b
Hypothalamus (VMH)	Central ceramide treatment with cell-penetrating C6 ceramides.	ER stress, sympathetic inhibition leading to reduced brown adipose tissue thermogenesis and weight gain.	Contreras et al., 2014
Hypothalamus (ARC)	CPT-1c overexpression increases ceramide levels; CPT-1c decreased ceramide levels.	Ceramide *de novo* synthesis mediates leptin anorexigenic action on feeding, downstream of malonyl-Co1 and CPT-1c.	Gao et al., 2011
Hypothalamus (mediobasal)	Ghrelin elicits a marked increase in C18:0 ceramides.	Ceramide *de novo* synthesis mediates ghrelin orexigenic action.	Ramirez et al., 2013

ceramide biosynthesis as a potential molecular mechanism by which altered hippocampal TG hydrolysis may affect energy balance. Ceramide content is increased upon LPL inhibition, and pharmacological inhibition of the *de novo* ceramide biosynthesis pathway is sufficient to prevent body weight gain and the associated phenotype in these animals (Picard et al., 2014b).

Recently, Cansell et al. (2014) showed that chronic brain TG delivery rapidly reduced both spontaneous and amphetamine-induced locomotion, abolished preference for palatable food, and reduced the motivation to engage in food-seeking behavior. Conversely, targeted disruption of the TG-hydrolyzing enzyme LPL specifically in the nucleus accumbens (area involved in cognitive processing of aversion, motivation and reward) increased feeding and food seeking behavior. Prolonged TG perfusion resulted in a return to normal palatable food preference despite continued locomotor suppression, suggesting that adaptive mechanisms occur (Cansell et al., 2014). Overall these results firmly establish that central hydrolysis of nutritional TG can be detected by the mesolimbic system through a LPL dependent mechanism, modulate the brain reward system and promote a state of craving for palatable food, and reduced energy expenditure associated with lower physical activity (two core mechanisms in the etiology of obesity). However, the inner mechanism relaying LPL action is not known, and it is likely to consider that, in the absence of exogenous lipids coming from LPL activity, lipogenesis and subsequent ceramide accumulation with ER stress, could be implicated (Weinstock et al., 1997; Wagner et al., 2004) and thus control food preference and reward seeking behavior.

CERAMIDE METABOLISM AS A TARGET FOR METABOLIC DISEASES?

A recent study combining lipidomic analysis in mouse models of obesity and in human prospective cohorts evidenced that plasma ceramides were diabetes susceptibility biomarker candidates (Wigger et al., 2017). A deep molecular analysis of the role of ceramide metabolism will help to understand the precise role of these sphingolipids in metabolic disease at the brain levels. As a number of pharmacological targets exists for ceramide reduction in pre-clinical studies, and some medications which inhibit ceramide production are currently approved for human use (Kornhuber et al., 2010), novel therapies targeting ceramide accumulation in brain (and peripheral tissues) may represent the future of obesity management and a better prevention of T2D. In particular ASM inhibitors hold promise for new therapies for Alzheimer's disease and depression, while acid ceramidase inhibitors are studied for cancer therapies review in Kornhuber et al. (2010). Pushing ceramide metabolism toward the synthesis of less harmful lipids, such as Sphingosine 1-phosphate, with the use of sphingosine kinase 1 activators could also represent a new therapeutic approach to counteract lipotoxicity (Bellini et al., 2015).

CONCLUSIONS

In conclusion, recent data evidenced that ceramides accumulation in brain under lipotoxic conditions might play a role on the deregulation of energy balance and lead to food intake disorders, obesity and the associated perturbation of glucose homeostasis (**Table 1**). Despite this evidence, the extent and consistency of ceramides effects in specific brain areas, and in particular the specificity of action from various ceramide species, needs to be clarified. Therefore, a better knowledge of ceramide action in brain may lead to earlier and more successful diagnoses and therapeutic options for patients suffering of obesity and associated metabolic disorders.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Mitochondrial Ultrastructure and Glucose Signaling Pathways Attributed to the Kv1.3 Ion Channel

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Gene-targeted deletion of the potassium channel Kv1.3 ($Kv1.3^{-/-}$) results in "Super-smeller" mice with a sensory phenotype that includes an increased olfactory ability linked to changes in olfactory circuitry, increased abundance of olfactory cilia, and increased expression of odorant receptors and the G-protein, G_{olf}. $Kv1.3^{-/-}$ mice also have a metabolic phenotype including lower body weight and decreased adiposity, increased total energy expenditure (TEE), increased locomotor activity, and resistance to both diet- and genetic-induced obesity. We explored two cellular aspects to elucidate the mechanism by which loss of Kv1.3 channel in the olfactory bulb (OB) may enhance glucose utilization and metabolic rate. First, using *in situ* hybridization we find that Kv1.3 and the insulin-dependent glucose transporter type 4 (GLUT4) are co-localized to the mitral cell layer of the OB. Disruption of Kv1.3 conduction via construction of a pore mutation (W386F Kv1.3) was sufficient to independently translocate GLUT4 to the plasma membrane in HEK 293 cells. Because olfactory sensory perception and the maintenance of action potential (AP) firing frequency by mitral cells of the OB is highly energy demanding and Kv1.3 is also expressed in mitochondria, we next explored the structure of this organelle in mitral cells. We challenged wildtype (WT) and $Kv1.3^{-/-}$ male mice with a moderately high-fat diet (MHF, 31.8 % kcal fat) for 4 months and then examined OB ultrastructure using transmission electron microscopy. In WT mice, mitochondria were significantly enlarged following diet-induced obesity (DIO) and there were fewer mitochondria, likely due to mitophagy. Interestingly, mitochondria were significantly smaller in $Kv1.3^{-/-}$ mice compared with that of WT mice. Similar to their metabolic resistance to DIO, the $Kv1.3^{-/-}$ mice had unchanged mitochondria in terms of cross sectional area and abundance following a challenge with modified diet. We are very interested to understand how targeted disruption of the Kv1.3 channel in the OB can modify TEE. Our study demonstrates that Kv1.3 regulates mitochondrial structure and alters glucose utilization; two important metabolic changes that could drive whole system changes in metabolism initiated at the OB.

Keywords: mitochondria, olfactory bulb, diet-induced obesity, glucose transporter, potassium channel

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INTRODUCTION

There are multifarious roles for potassium channels beyond being drivers of the resting potential (Kaczmarek, 2006), although classically potassium channels are dampeners of excitability through timing of the interspike interval and shaping of the action potential (AP; Jan and Jan, 1994; Yellen, 2002). Among the non-conductive functions of potassium channels lies their ability to detect, be regulated, or be modulated by energy substrates or metabolites, namely glucose, ATP, or NADPH (Pan et al., 2011; Tucker et al., 2013; Tinker et al., 2014). This original research article will present a review of metabolic sensing by a brain region outside the traditional endocrine axis controlling food intake and energy balance—the olfactory bulb (OB). We highlight the role of a particular potassium channel, Kv1.3, which is highly expressed in the OB and may serve as the molecular sensor of metabolism. We hypothesize that the olfactory system has a dual function, to transduce external chemical signals, or odorants, into an internal representation, and simultaneously to detect internal chemistry, namely metabolically important molecules, such as glucose and insulin (Fadool et al., 2000, 2011; Tucker et al., 2013). Thus, the OB is a detector of metabolic state; the biophysical properties of Kv1.3 are modulated by energy availability and the disruption of energy homeostasis can modify olfactory sensory coding (Palouzier-Paulignan et al., 2012). Such metabolic sensing could change food intake given the intimate relationship of olfactory perception to satiety and food choice (Aime et al., 2007; Tong et al., 2011; Badonnel et al., 2014; Soria-Gomez et al., 2014; Lacroix et al., 2015). We summarize current data leading to the glucose-sensing ability of Kv1.3-expressing neurons in the OB and present new data showing the expression of glucose transporter type 4 (GLUT4) in the OB. We report biochemical evidence that Kv1.3 activity can alter GLUT4 translocation, and demonstrate ultrastructural changes in the OB mitochondria of Kv1.3$^{-/-}$ mice. To place our new studies in context, we will (1) describe the phenotype of Kv1.3$^{-/-}$ mice and the role of this voltage-dependent channel in olfaction, (2) discuss the potential therapeutic benefits of targeting Kv1.3 channels centrally and peripherally for energy homeostasis, (3) present the known distribution and role of glucose transporters in the OB, (4) summarize the known expression of Kv1.3 in mitochondria, and (5) conclude the introduction by describing recent data that demonstrate the impact of metabolic imbalance on the structure/function of the OB.

The discovery of the many non-conductive roles for Kv1.3 was made through loss of function studies using a whole-animal, targeted deletion of the Kv1.3 gene (Koni et al., 2003). We and other laboratories identified the Kv1.3$^{-/-}$ mice as thinner than their wild-type (WT) counterparts without caloric self-restriction (Xu et al., 2003, 2004; Fadool et al., 2004). The mice had lower plasma fasting glucose levels, lower leptin levels, and had greater insulin sensitivity when challenged with an intraperitoneal glucose tolerance test (Xu et al., 2003; Tucker et al., 2008, 2010; Thiebaud et al., 2014). Using a custom-designed metabolic chamber to quantify systems physiology parameters and ingestive behaviors (Williams et al., 2003), we found that the Kv1.3$^{-/-}$ mice ate frequent small meals and drank large boluses of water while still maintaining identical total caloric and water intake as that of WT animals (Fadool et al., 2004). Kv1.3$^{-/-}$ mice showed slightly elevated metabolic activity (Xu et al., 2003; Fadool et al., 2004) and increased locomotor activity, particularly in the dark cycle (Fadool et al., 2004). Interestingly, Hennige et al. (2009) demonstrated that an i.c.v. injection of the Kv1.3 pore blocker, margatoxin, similarly elevated locomotor activity and increased cortical AP frequency. Recently when provided access to voluntary running wheels, the Kv1.3$^{-/-}$ mice had no marked irregularity in circadian rhythm. The mice ran the same daily distance as WT mice, but they ran at a lower velocity and in more frequent exercise bursts (Gonzalez et al., unpublished data). When examined for olfactory acuity, Kv1.3$^{-/-}$ mice showed a heightened sense of smell in terms of both odorant discrimination and odor threshold (Fadool et al., 2004). The Kv1.3 channel carries 60–80% of the outward current in mitral cells of the OB (Fadool and Levitan, 1998), which are the first order processing cells for olfactory information. Not unexpectedly, deletion of Kv1.3$^{-/-}$ in these neurons elicits an increase in the evoked firing frequency, attributed to more spikes/AP cluster, a shortened interburst interval, and also a depolarizing shift in the resting membrane potential (Fadool et al., 2004, 2011). The olfactory circuitry of Kv1.3$^{-/-}$ mice has a smaller number of axonal projections from the olfactory epithelium to identified synaptic targets, or glomeruli, and the projection is not mistargeted. The glomeruli, however, are smaller and supernumerary (Biju et al., 2008). Finally, the expression of G-protein coupled odor receptors (ORs) is enhanced in the Kv1.3$^{-/-}$ mice, with a concomitant increase in OR density in the ciliary processes that contain this odorant transduction machinery (Biju et al., 2008). This collective phenotype in the knockout mice reveals the broad span of non-conducting functions of Kv1.3 channels, from energy homeostasis, to ingestive behavior, to axonal targeting, and sensory perception or odor tuning.

Because Kv1.3$^{-/-}$ mice exhibited altered ingestive behaviors (Fadool et al., 2004), we and others challenged them with modified-fat diets and discovered a resistance to diet-induced obesity (DIO) (Xu et al., 2003; Tucker et al., 2012). Moreover, when Kv1.3$^{-/-}$ mice were bred to homozygosity with melanocortin-4 receptor-null mice (Tucker et al., 2008), which have a disruption of the hypothalamic, anorexogenic pathway

Abbreviations: AP, action potential; ATP, adenosine triphosphate; AVMA, American Veterinary Medical Association; BSA, bovine serum albumin; CF, control diet; CMV, cytomegalovirus; ddH_2O, double-distilled water; DIO, diet-induced obesity; EtOH, ethanol; FSU, Florida State University; GCCP, Guidance on Good Cell Culture Practices; GCL, granule cell layer; GLUT4, glucose transporter type 4; h, hour; HEK, human embryonic kidney; IACUC, Institutional Animal Care and Use Committee; IMM, inner mitochondrial membrane; IP, immunoprecipitation; IR, insulin receptor; Kv1.3, voltage-dependent potassium channel 1.3; MEM, minimal essential medium; MHF, moderately high-fat diet; min, minute; ML, mitral cell layer; NADPH, nicotinamide adenine dinucleotide phosphate; NIH, National Institutes of Health; NTMT, alkaline phosphatase buffer; OB, olfactory bulb; OMM, outer mitochondrial membrane; OR, olfactory receptor; OSN, olfactory sensory neuron; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline + Tween; PFA, paraformaldehyde; PG, periglomerular; PKA, protein kinase A; PKC, protein kinase C; PPI, phosphatase inhibitor; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; *snk*, Student Newman Keuls *post-hoc* test; SSC, saline sodium citrate; TEE, total energy expenditure; WT, wildtype.

and are a model for genetic obesity, the resultant progeny had reduced adiposity and body weight, attributed to increased locomotor activity and energy expenditure. Interestingly, while Kv1.3 has a selective distribution within the central nervous system (dentate gyrus, OB, and olfactory cortex; Kues and Wunder, 1992), its biophysical properties were first characterized in T lymphocytes (Cahalan et al., 1985). Others have explored the channel's capacity to regulate either body weight or insulin sensitivity (Xu et al., 2004; Upadhyay et al., 2013) by blocking channel activity in the periphery. Intraperitoneal injection of margatoxin, which blocks the vestibule of the Kv1.3 channel (Knaus et al., 1995), was found to increase insulin sensitivity in wildtype and genetic models of obesity, namely ob/ob and db/db mice (Xu et al., 2004). A similar effect was discovered as a result of a different channel blocker, ShK186, which was effective in increasing peripheral insulin sensitivity in mice that were fed an obesogenic diet of high fat and fructose (Upadhyay et al., 2013). Beyond enhanced insulin sensitivity in DIO mice, ShK-186 treatment also reduced weight gain, adiposity, and fatty liver and activated brown fat to augment oxygen consumption and energy expenditure. Although there have been conflicting reports on the ability of the channel to improve insulin sensitivity based upon the pharmacological inhibition of peripheral Kv1.3 across different skeletal muscle cell lines (Straub et al., 2011; Hamilton et al., 2014) and exploration of the selectivity of Kv1.3 blockers themselves (Bartok et al., 2014), peripheral targeting of the Kv1.3 channel remains a strong therapeutic target for regulating diabetes, inflammation, and other diseases (Choi and Hahn, 2010; Pérez-Verdaguer et al., 2015).

One manner by which Kv1.3 is thought to promote insulin sensitivity and glucose uptake is through the insulin-sensitive glucose transporter type 4 (GLUT4) (Xu et al., 2004). Inhibition or gene-targeted deletion of Kv1.3 stimulates glucose uptake and translocation of GLUT4 in adipose tissue and skeletal muscle. Because glucose ($C_6H_{12}O_6$) is a polar molecule, its transport across the plasma membrane requires integral transport proteins. Carriers for glucose were first isolated from the membranes of HepG2, a hepatic cell line, as reviewed by Baly and Horuk (1988). Since then, different families and classes of glucose transporters have been identified in peripheral tissues as well as in the brain. Glucose is transported across biological membranes via glucose transporters (GLUTs) according to "an alternating conformer model." According to this model, glucose can bind to one of two mutually-exclusive binding sites of the transporter, either on the extracellular or the intracellular site. The glucose transporter switches from one conformation to another to release its substrate (Carruthers, 1990). GLUTs comprise 14 family members named in the order they were cloned, and divided into three subfamilies: class I (GLUT1-4, and GLUT14), class II (GLUT5, GLUT7, GLUT9, and GLUT11), and class III (GLUT6, 8, 10, 12, and the myoinositol transporter HMIT1 or GLUT13) (see **Table 1**). GLUTs exhibit diverse substrate and tissue specificity, resulting in distinct functional characteristics. Their sequences, however, are similar in that (1) 12 helices span the plasma membrane with seven conserved glycine residues in each helix, (2) the intracellular surface has several basic and acidic residues, and (3) two tryptophan and two tyrosine residues are conserved (Joost and Thorens, 2001).

It has been shown that loss of Kv1.3 translocates GLUT4, a member of the family of facilitative glucose transporters (GLUTs) or class I, in a calcium-dependent and insulin-independent manner (Xu et al., 2004; Desir, 2005). In adipocytes, the mechanism of translocation is thought to occur through a PI3K-independent pathway consisting of depolarizing the plasma membrane and increasing the intracellular Ca^{2+} concentration (Li et al., 2006). The co-localization of GLUT4 and Kv1.3 centrally has not been explored and the mechanism of glucose signaling in the OB has only recently been investigated (Tucker et al., 2013; Al Koborssy et al., 2014) for such an energy demanding process as odor processing (Carter and Bean, 2009; Lecoq et al., 2009, 2011). Kv1.3 currents can be modulated by metabolically active (d-glucose) rather than inactive (l-glucose) glucose in a dose-dependent manner that follows a bell-shaped curve (Tucker et al., 2013). Using a slice preparation of the OB and current-clamp configuration, we found that mitral cells of the OB could be either excited or inhibited by glucose (Tucker et al., 2013). The change in mitral cell excitability was linked to a changed latency to first spike and alternation of the AP cluster length. Kv1.3$^{-/-}$ mice were insensitive to glucose and unlike modulation by phosphorylation, glucose-induced change in mitral cell activity was rapid and reversible within the time course of a patch recording. We now report the presence of GLUT4 and Kv1.3, centrally, co-localized in the mitral cell layer of the OB. Moreover, using a heterologous expression system (HEK 293 cells), we are able to increase the translocation of GLUT4 when the pore of Kv1.3 (Delaney et al., 2014) is mutated to yield a non-conducting channel. This suggests that a number of modulators of Kv1.3 that can inhibit channel conductance (src, EGF, insulin, BDNF, glp-1, Nedd4-2) (Holmes et al., 1996; Bowlby et al., 1997; Fadool et al., 1997, 2000; Fadool and Levitan, 1998; Cook and Fadool, 2002; Colley et al., 2009; Thiebaud et al., 2016; Velez et al., 2016), could be poised to increase GLUT4 translocation and the utilization of glucose by mitral cells of the OB.

While Kv1.3 channels do not exist in isolation in the plasma membrane—they are part of a well-characterized scaffold of proteins and adaptor proteins that act to interface signaling cascades with the channel (Cook and Fadool, 2002; Marks and Fadool, 2007; Colley et al., 2009)—the channels are not solely restricted to this organelle. The channel is additionally highly expressed in the inner mitochondrial membrane, or IMM, of several cell types including lymphocytes (Szabo et al., 2005), cancer cells (Leanza et al., 2012), and hippocampal neurons (Bednarczyk et al., 2010). It is for this reason, and given the metabolic and olfactory phenotypes of Kv1.3$^{-/-}$ mice, that we undertook an ultrastructural analysis of mitochondria in the OB. The IMM is home to many types of ion channels and exchangers for a variety of cations and anions (Bernardi, 1999), which maintain a negative membrane potential of the organelle. The tightly controlled IMM permeability is critical for efficient ATP production and a dysregulation of IMM permeability often leads to cell death (Kim et al., 2003). It has been proposed that potassium ion channels play an important role in the control of the integrity of the IMM (Szewczyk et al., 2009) whereby

TABLE 1 | Distribution of glucose transporters and Kv1.3 channel.

	Transporter/Channel	Experimental Model	Technique	Localization	References
CLASS I	GLUT1	Mouse	IHC (gold particles)	Asymmetric expression in cortex, hippocampus, cerebellum, and OB	Dobrogowska and Vorbrodt, 1999
	GLUT1 and GLUT2	Mouse	IHC and *in situ* hybridization	Tanycytes in hypothalamus	García et al., 2003
	GLUT1, GLUT3, and GLUT4	Bovine, Rat	Western blot, qPCR, and ICC	Monocytes, olfactory bulb	O'Boyle et al., 2012; Al Koborssy et al., 2014
CLASS II	GLUT 5	Rat	RNA blotting	Small intestine, kidney, and brain	Rand et al., 1993
	GLUT7	Human, Rat	Northern blot, IHC, and Western blot	Small intestine, colon, testes, and prostate in humans. Small intestine and low expression in colon of rat	Cheeseman, 2008
	GLUT9	Human	Northern blot	Highly expressed in kidney and liver. Low levels of expression in lung, leukocytes, & heart	Phay et al., 2000
	GLUT11	Human, *Xenopus* oocytes	Northern blot and qPCR	Three splice variants: GLUT11-A in heart, skeletal muscle and kidney. GLUT11-B in kidney, adipose, and placenta. GLUT11-C in adipose, heart, skeletal muscle, and pancreas.	Scheepers et al., 2005
CLASS III	GLUT10 and GLUT12	Human, Mouse	RT-PCR	Adipose tissue: mature adipocytes and stromal vascular cells	Wood et al., 2003
	GLUT8	Mouse, Rat, Human	Northern blot	Highly expressed in human testes and sperm cells. Low amounts detected in several brain areas, skeletal muscle, heart, and small intestine	Schmidt et al., 2009
	GLUT13 (HMIT1)	Mouse, *Xenopus* oocytes	Western blot, Northern blot, Immunofluorescence	Multiple brain areas; mostly in glial cells but also present in neurons	Cura and Carruthers, 2012
CHANNEL	Kv1.3	Human cell line	Patch-clamp electrophysiology	Voltage-gated potassium channel first characterized in T-lymphocytes	Cahalan et al., 1985
	Kv1.3	Rat	*In situ* hybridization	Dentate gyrus, OB, piriform cortex	Kues and Wunder, 1992
	Kv1.3	Human and mouse cell lines	Western blot	T-lymphocytes	Cai and Douglass, 1993
	Kv1.3	Rat cell culture, Mouse, Rat	ICC and Patch-clamp electrophysiology	Kv1.3 highly expressed in mitral cells of olfactory bulb	Fadool and Levitan, 1998; Fadool et al., 2000, 2004
	Kv1.3	Rat	IHC, RT-PCR, immunofluorescence, and immunoblotting	Kv1.3 expressed in both cortex and medulla of kidney	Carrisoza-Gaytán et al., 2010

IHC, immunohistochemistry; ICC, immunocytochemistry; OB, olfactory bulb; RT-PCR, reverse transcriptase PCR; qPCR, quantitative PCR.

substantial evidence has shown cardio- and neuro-protective effects of targeting mitochondrial potassium channels (Szewczyk and Marban, 1999; Busija et al., 2004; O'Rourke, 2004). The mechanism of these effects has been linked to the mitochondrial production of reactive oxygen species or ROS (Malinska et al., 2010). Interestingly, it has been shown that oxygen/glucose deprivation of microglia results in suppression of Kv1.3 currents in a tyrosine phosphorylation signaling cascade, which can be significantly attenuated by ROS scavengers (Cayabyab et al., 2000). It is possible that mitochondrial Kv1.3 functions could go beyond cytoprotection and involve ROS-dependent energy metabolism. ROS signaling is usually considered to be detrimental and damage-promoting, however, ROS can act as signaling molecules regulating organismal homeostasis, stress responsiveness, health, and longevity (Shadel and Horvath, 2015). ROS production is linked to the central control of whole body metabolism (Shadel and Horvath, 2015). In fact, there is considerable evidence showing that ROS can enhance insulin sensitivity by oxidizing multiple signaling molecules (Mahadev et al., 2001, 2004; Loh et al., 2009; Cheng et al., 2010), while long-term excessive ROS may cause insulin resistance (Szendroedi et al., 2012).

Metabolic imbalance attributed to DIO has been shown to disrupt the structure and function of the olfactory system. When mice were challenged with high-fat or high-fat, high-carbohydrate diets, the excess energy imbalance resulted in marked loss of olfactory sensory neurons (OSNs), loss of axonal projections to defined glomerular targets, reduced electro-olfactogram amplitude, irregular AP firing frequency in mitral cells of the OB, reduced olfactory discrimination ability, slowed reward-reinforced behaviors, and disrupted reversal learning (Fadool et al., 2011; Thiebaud et al., 2014). Insulin-induced Kv1.3 phosphorylation in the OB (Fadool and Levitan, 1998; Fadool et al., 2000) was lost in DIO mice (Marks et al., 2009) and slices from these mice exhibited loss of insulin modulation of mitral cell excitability, exemplifying a degree of insulin resistance for mitral cell function in the obese state (Fadool et al., 2011). Obesity-resistant Kv1.3$^{-/-}$ mice exhibited similar loss of OSNs to that of WT mice while challenged with high-fat, high-carbohydrate diet, but not when fed a high-fat diet. The Kv1.3$^{-/-}$ mice showed a concomitant loss of olfactory discrimination and slowed reward-reinforced behaviors only when challenged with the high-fat, high-carbohydrate diet. Kv1.3$^{-/-}$ mice oddly showed improved olfactory discrimination with the high-fat diet and showed reversal learning capacity regardless of diet.

Herein, in an effort to further elucidate the mechanism of the metabolic phenotype of Kv1.3$^{-/-}$ mice, we report the presence of GLUT4 in the same OB neurolamina as the Kv1.3 channel and explore the ultrastructure of mitochondria in the Kv1.3$^{-/-}$ mice.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Florida State University (FSU) under protocols #1124 and #1427, and followed the guidelines set forth by the National Institutes of Health (NIH) and the American Veterinary Medical Association (AVMA). Mice were anesthetized with isoflurane (Aerrane; Baxter, Deerfield, IL, USA) using the IACUC-approved drop method and were then sacrificed by decapitation (AVMA Guidelines on Euthanasia, June 2007).

Solutions

Phosphate-buffered saline (PBS) contained (in mM): 136.9 NaCl, 2.7 KCl, 10.1 Na_2HPO_4, and 1.8 KH_2PO_4 (pH 7.4). Phosphate-buffered saline + Tween (PBST) contained 1x PBS and 0.1% Tween. Hybridization buffer contained 0.5 M formamide, 0.25 M 20x SSC, 5 mg/mL torula yeast RNA, 50 mg/mL heparin sodium salt, and 0.1% Tween. 20x SSC contained 3 mM sodium chloride and 342 μM sodium citrate (pH 7.2). Blocking solution for *in situ* hybridization contained 0.2% BSA in PBST. NTMT contained (in mM): 100 Tris-HCl (pH 9.5), 25 $MgCl_2$, 100 NaCl, and 0.1% Tween 20 (Cold Spring Harbor Protocols; http://cshprotocols.cshlp.org/). Wash buffer contained (in mM); 25 Tris, 250 NaCl, 5 EDTA, and 0.1% Triton X-100 (pH 7.5). Phosphatase inhibitor mixture (PPI) contained 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin. All salts and reagents were obtained from Sigma Chemical (St Louis, MO, USA) or Fisher Scientific, Inc. (Atlanta, GA, USA) unless otherwise stated.

Animal Care

All mice used in our study were adult C57BL/6J male mice (000664 | Black 6; The Jackson Laboratory, Bar Harbor, ME, USA). Mice were weaned at postnatal day 29 and singly housed in conventional open cages at the FSU vivarium under a 12/12 h light/dark cycle with *ad libitum* access to food and water. For ultrastructural studies, wild-type (WT) and Kv1.3$^{-/-}$ mice (Xu et al., 2003; Fadool et al., 2004) were maintained on either a control diet (CF; Purina 5001 Rodent Chow; 28.05% kcal protein, 59.81% kcal carbohydrate, and 13.5% kcal fat; Richmond,VA, USA) or a moderately high-fat condensed milk diet (MHF; Research Diets D12266B; 16.8% kcal protein, 51.4% kcal carbohydrate, and 31.8% kcal fat; New Brunswick, NJ, USA) for a duration of 4 months.

In situ Hybridization

RNA Probes

RNA from the OB of WT and Kv1.3$^{-/-}$ mice was isolated with Trizol reagent (Life Technologies, CA, USA). Total RNA was phenol/chloroform extracted, precipitated with isopropanol, washed with ethanol, and then dissolved in ultra-pure distilled water. Total cDNA was obtained by reverse transcription with oligo dT primer (Invitrogen, Carlsbad, CA, USA). Targeted genes were amplified by polymerase chain reaction using the following primers: GLUT4 primers forward 5′-TCTCAGCTGCCTTCCGAC-3′ and reverse 5′-TACCAAAGGCTCCCTCCC-3′; Kv1.3 primers forward 5′-CTTCGACGCCATCCTCTACTAC-3′ and reverse 5′-AAGCTCAAAGGAGAACCAGATG-3′; TH primers forward 5′-GATTGCAGAGATTGCCTTCC-3′ and reverse 5′-CCTGTGGGTGGTACCCTATG-3′. Kv1.3 and GLUT4 amplicons were cloned into pGEMT (Promega, Madison, WI, USA). The vector was linearized with *Sac* I for Kv1.3 and *Xba* I for GLUT4. Digoxigenin-UTP labeled RNA probes were made according to the manufacturer's specifications (Roche, Indianapolis, IN, USA) using SP6 RNA polymerase. Tyrosine hydroxylase (TH) RNA probe was generated as a positive control (result not shown) (Cubells et al., 1995), and Kv1.3 probe was applied to slices of Kv1.3$^{-/-}$ mice as a negative control.

In situ Hybridization

WT and Kv1.3$^{-/-}$ mice were perfused with 4% paraformaldehyde (PFA), and the OBs were quickly dissected and placed in the perfusion solution overnight at 4°C. Thirty- micron slices were cut on a microtome (Leica CM1850, Buffalo Grove, IL, USA) and were left free floating in 100% methanol until use. For labeling, free-floating slices were transferred to a nine-well glass plate. Slices were first re-hydrated in a graded methanol series diluted in PBS (75, 50, and 25%) for 5 min each then 4 times with 100% PBST 0.1% for 5 min each. Slices were digested with 10 mg/mL proteinase K dissolved in PBST for 15 min, followed by a 10-min rinse in PBST, a 20-min fixation with PFA, and five rinses of 5 min each in PBST. Slices were pre-hybridized in hybridization buffer for 2 h at 60 °C, and then hybridized

overnight with the probe at 60°C. Probe concentration was 0.5 ng/μL and total volume applied was 300 μL. The next day, slices were first rinsed at 60°C with graded dilutions of 2x SSC in hybridization buffer (25, 50, 75, and 100%) 10 min each, then twice at 68°C with 100% 0.2x SSC for 30 min, followed by 5-min rinses with graded dilutions (75, 50, 25, and 0%) of 0.2x SSC in PBST. PBST was then removed and replaced with blocking solution for 2 h at 4°C. Anti-digoxigenin Fab fragment conjugated to alkaline phosphatase was added overnight at 4°C. On day 3, slices were washed with blocking buffer then NTMT. DIG-labeled RNA probes were immunodetected with DIG Nucleic Acid Detection Kit (NBT/BCIP) according to the manufacturer's protocol (Roche). Slices were finally mounted on microscopic slides before visualization.

Electron Microscopy

All electron microscopy reagents were purchased from Electron Microscopy Sciences (EMS; Hatfield, PA, USA) unless otherwise noted. Following maintenance on modified diets, mice were perfused through the heart with PBS then with 4% PFA. OBs were quickly dissected and fixed in 4% PFA overnight, followed by infiltration with 10, and 30% sucrose in PBS at 4°C. Coronal OB sections were cut to 100 μm thickness on a vibratome (Leica Model 1000, Wetzlar, Germany), and transferred to a 24-well plate. Slices were washed three times with PBS, and then were incubated in 2% osmium tetroxide at room temperature for an hour. This was followed by a series of graded alcohol washes with 50% ethanol (EtOH) and 70% EtOH for 10 min each. The sections were further incubated with 1% uranyl acetate in 70% EtOH for 1 h before being dehydrated sequentially in 70, 90 and twice in 100% EtOH for 10 min each. After dehydration, sections were washed twice in propylene oxide for 10 min after which they were embedded in EMbed 812 Resin /propylene oxide (1:1) overnight on a rotary shaker in a fume hood to allow the propylene oxide to gradually evaporate. The following day, sections were infiltrated twice with fresh Epon for 12 h each on a shaker. Next the sections were flat embedded in fresh Epon between ACLAR plastic sheets (EMS) and polymerized in a 60°C oven over 3 days. ACLAR sheets were examined with a Nikon SMZ1000 stereoscopic microscope (Melville, NY, USA) and areas of the Epon film containing stretches of intact OB tissue layers were excised and remounted on Epon blocks with Scotch Super Glue Liquid (3M). Silver-gold sections (90–100 nm) were cut with a Leica Ultracut E microtome (Buffalo Grove, IL, USA) and mounted on 3.05 mm copper hexagonal mesh grids. The grids were post-stained with 2% uranyl acetate in ddH_2O for 10 min followed by 4% Reynold's lead citrate for 5 min. Images were recorded on a FEI CM120 transmission electron microscope (Hillsboro, OR, USA) using a Tietz Tem-Cam F224 slow scan CCD camera. Mitochondrial counts were taken from a series of low magnification electron micrographs totaling ~2000 μm^2 for each experimental condition. Within each 51.5 μm^2 field of view, all mitochondria were counted. Mitochondrial area and circularity ($4\pi \times$ area / perimeter^2) were quantified using Photoshop CS4 software (Adobe, San Jose, CA, USA). Approximately 40 fields of view from each experimental condition ($CF^{+/+}$, $MHF^{+/+}$, $CF^{-/-}$, $MHF^{-/-}$) were analyzed as sampled from 3 mice in each condition.

cDNA Constructs

The expression vectors were mammalian-based and contained the cytomegalovirus (CMV) promotor upstream from the coding region. Kv1.3 was ligated into the Invitrogen vector $pcDNA_3$ at the unique *Hind* III site of the multiple coding region as previously described (Fadool et al., 2000). A non-conducting mutant Kv1.3 channel (W386F Kv1.3) was generated by site-directed mutagenesis as previously described (Holmes et al., 1997). The cDNA for GLUT4-myc-eGFP was generously provided by Dr. Jeffrey E. Pessin (Albert Einstein College of Medicine, Bronx, NY, USA).

Cell Maintenance and Transient Transfection

Human embryonic kidney (HEK) 293 cells were handled according to the guidelines of the European Center for the Validation of Alternative Methods Task force based on the Guidance on Good Cell Culture Practices (GCCP; Coecke et al., 2005). Cells were grown at 37°C in modified Eagle's minimum essential media (MEM; Gibco/Life Technologies, Carlsbad, CA, USA) supplemented with 2% penicillin/streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (Gibco/Life Technologies). Cells were grown to 75–80% confluency for 3–4 days, after which cDNA was introduced with lipofectamine (Thermo Fisher Scientific, #18324-012; Waltham, MA, USA) as previously described (Marks and Fadool, 2007). Briefly, 3 μg of each cDNA construct was complexed with 18 μl of lipofectamine for 30 min in Opti-MEM (Gibco), after which the cDNA/lipofectamine/Opti-MEM mixture was applied to HEK293 cells in a 60 mm dish for 5 h before the transfection was stopped by replacing with MEM. Proteins were immunoprecipitated 2 days post transfection.

Immunoprecipitation and Western Blot

Cells were lysed and prepared for immunoprecipitation following a modification of procedures by Colley et al. (2007). Two days post transfection, HEK 293 cells designated for detection of surface GLUT4 were first incubated for 30 min at 37°C with 1:300 c-myc antibody (9E10 clone, Roche), then washed three times with sodium orthovanadate diluted in PBS. Cells were then lysed in ice-cold PPI mixture for 30 min. The lysates were clarified by centrifugation at 14,000 g for 10 min at 4°C and the supernatant rotated overnight at 4°C on a Roto-Torque slow speed rotary (model 7637; Cole Palmer, Vernon Hills, IL). The immunoprecipitated protein harvested the next day with 3 mg/ml Protein A-Sepharose (GE Healthcare, Pittsburgh, PA, USA). To collect total GLUT4, cells were lysed first with PPI and then precleared with protein Sepharase A for 3 h before anti-myc (1:400; 9E10 clone, Roche) was added overnight to the whole-cell lysate. The next day, protein Sepharose A was added again to the immunoprecipitated proteins for 3 h, followed by 4 wash steps using centrifugation at 14,000 g (4°C) for 10 min each with wash buffer (see section Solutions).

Immunoprecipitated proteins from whole-cell or surface detection of GLUT4 were separated on 10% acrylamide sodium dodecyl sulfate (SDS) gels and electro-transferred to nitrocellulose for Western blot analysis as previously described (Colley et al., 2007). Blots were blocked with 1% nonfat dry milk (Biorad Laboratories, Hercules, PA, USA), and incubated overnight at 4°C in primary antibody against c-myc (1:700) then with secondary antibody (goat anti-mouse; 1:3000; Sigma A2304) for 90 min at room temperature. Protein bands were visualized using enhanced chemiluminescence Western blotting detection reagent (ECL; GE Healthcare) exposure on Fuji RX film (Fisher Scientific). A Hewlett-Packard Photosmart Scanner (model 106–816, Hewlett Packard, San Diego, CA) was used in conjunction with Quantiscan software (Biosoft, Cambridge, England) to quantify the densitometry of protein bands revealed on the film autoradiographs.

Statistical Analysis

All data are reported as the mean (± s.d.). Significantly-different means were determined by a one-way analysis of variance (ANOVA) due to multiple comparison design of all experiments. A Bonferoni *post-hoc* test was applied for protein biochemistry experiments and a Student Newman Kuels (*snk*) was applied for electron microscopy experiments. Different length vertical lines represent significantly different *post-hoc* comparison.

RESULTS

mRNA of GLUT4 and Kv1.3 are Co-localized in Mitral Cells of the Olfactory Bulb

We have previously demonstrated that mitral cells are glucose-sensitive and that modulation of AP firing frequency is dependent upon Kv1.3 (Tucker et al., 2010, 2013). GLUT4 mRNA is expressed in 75% of glucose-sensing neurons of the ventromedial nucleus of the hypothalamus (Kang et al., 2004). We therefore hypothesized that GLUT4 would be localized to the mitral cell layer where Kv1.3 channels carry 60–80% of the outward current in these primary output neurons (Fadool and Levitan, 1998). mRNA expression of GLUT4 was examined by *in situ* hybridization with digoxigenin-labeled riboprobes. A robust expression of GLUT4 transcripts was found in the soma of mitral cells within the mitral cell layer (ML) from WT mice (**Figure 1**). A faint labeling was observed in the granule cell layer (GCL) and a few periglomerular cells (PG) expressed GLUT4 mRNA. The expression pattern for GLUT4 was similar in Kv1.3$^{-/-}$ mice, suggesting that the loss of Kv1.3 channel did not affect transporter transcript expression. The Kv1.3 mRNA transcript was also robustly visualized in the ML of WT mice in nearly every mitral cell that was examined. Kv1.3 mRNA was also visualized in PG cells to a greater extent than that of the GLUT4 probe. An absence of Kv1.3 mRNA labeling was observed in Kv1.3$^{-/-}$ across all neural lamina (**Figure 1**, bottom). A similar absence of labeling was found for slices where the probes were omitted (data not shown).

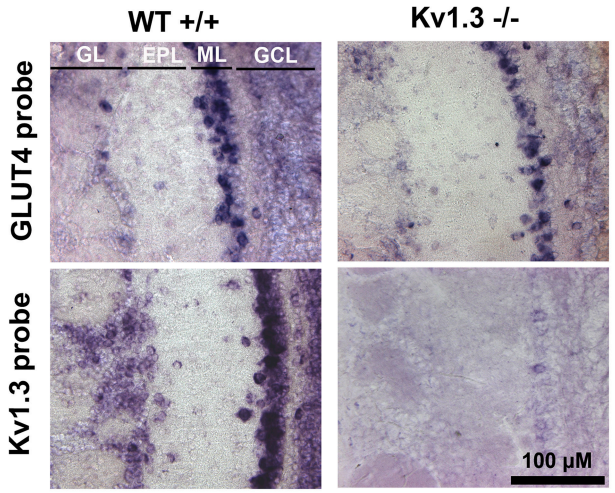

FIGURE 1 | Kv1.3 and GLUT4 mRNA are expressed predominantly in the glomerular and mitral cell layers of the olfactory bulb. Light photomicrographs containing tissue sections of wildtype (WT$^{+/+}$; left) and Kv1.3-null mice (Kv1.3$^{-/-}$; right) olfactory bulbs labeled with GLUT4 (GLUT4 probe; top) or Kv1.3 (Kv1.3 probe; bottom). Note that GLUT4 mRNA is expressed predominantly in the mitral cell layer (ML) in both WT and Kv1.3$^{-/-}$ mice, whereas Kv1.3 mRNA is localized to both the glomerular layer (GL) and ML. GL, glomerular layer; EPL, external plexiform layer; ML, mitral cell layer; GCL, granule cell layer.

GLUT4 Translocation is Facilitated by Blocking Kv1.3 Conductance

Mice with genetic deletion of Kv1.3 are resistant to modulation by glucose (Tucker et al., 2013). Kv1.3 serves as an insulin receptor (IR) kinase substrate, and its activity can be inhibited by tyrosine phosphorylation (Fadool et al., 2000). Xu et al. (2004) found that genetic or pharmacological blockade of Kv1.3 exerted the same effect as insulin whereby GLUT4 translocation to the membrane of adipocytes and skeletal muscles was increased. Kv1.3 inhibition enhances GLUT4 translocation in adipocytes through a PI3K-independent mechanism consisting of depolarizing the plasma membrane and increasing intracellular Ca^{2+} concentration (Li et al., 2006). To prove that Kv1.3 activation serves as molecular trigger of GLUT4 translocation to the membrane, HEK 293 cells were transiently transfected with the cDNA coding for GLUT4 plus wildtype or a pore mutant Kv1.3 channel. W386F Kv1.3 is a construct whereby a threonine is substituted for a phenylalanine and results in the translation of a non-conducting channel (Holmes et al., 1997; Delaney et al., 2014). Whole-cell or surface-labeled GLUT4 was visualized using anti-c-myc directed against a myc extracellular, epitope tag on GLUT4 (**Figure 2A**). Whole-cell lysates showed a strong label for total GLUT4 cell expression (**Figures 2B,C**). GLUT4 + Kv1.3 yielded a significantly lower total receptor cell expression that was increased when the W386F Kv1.3 was substituted in the transfection scheme [significantly different, one-way analysis of variance (ANOVA) with Bonferoni's *post-hoc* test, $F_{(2,18)} = 5.032$, $p = 0.0184$]. Immunoprecipitation (IP) was used to isolate the surface-labeled GLUT4 from total receptor cell expression (**Figures 2D,E**). The IP strategy revealed very little GLUT4 at

the plasma membrane during a static state, consistent with its known intracellular storage pool reported by Li et al. (2009). However, a consistent expression of GLUT4 was found in the Kv1.3 co-transfection condition and a strong increase in receptor expression was observed when Kv1.3 was substituted by W386F Kv1.3 [significantly different, one-way ANOVA with Bonferoni's *post-hoc* test, $F_{(2,12)} = 6.242$, $p = 0.0139$]. Our results indicate that changes in Kv1.3 conductance produced by pore mutagenesis upregulates GLUT4 translocation to the membrane surface. Such regulated receptor membrane availability would reflect the capacity to modify glucose utilization at any given time.

Kv1.3$^{-/-}$ Mice have Smaller Mitochondria that are Resistant to Morphological Changed Induced by Diet-Induced Obesity

Elevated plasma glucose after a meal activates mitochondrial oxidative phosphorylation in pancreatic cells to increase ATP release and insulin secretion. A chronic rise in glucose, such as in diabetic or obese patients, has caused mitochondrial atrophy, which suggests an important connection between mitochondria and metabolic imbalance (Gerbitz et al., 1996). Several potassium channels contribute to mitochondrial function including Kv1.3 (Bednarczyk, 2009). We decided to investigate the change in mitochondrial morphology in the OB of WT and Kv1.3$^{-/-}$ mice that have been challenged with a MHF vs. CF diet.

Electron micrographs showing the abundance and size of mitochondria examined within the cell bodies of mitral cells are found in **Figures 3A,B**, respectively (low and higher magnification). The number, cross-sectional area, and circularity index of mitochondria from 40 fields of view were quantified from three mice in each of the four conditions (**Figures 3C–E**). For control fed mice, there was no change in the number of mitochondria across the genotypes [number: one-way ANOVA, $F_{(3,\ 161)} = 4.925$, $p = 0.0027$, Student Newman Keuls's (*snk*) *post-hoc* test CF$^{+/+}$ vs. CF$^{-/-}$, $p \geq 0.05$], however the cross-sectional area was larger and circularity index was less in the WT mice compared with that of the Kv1.3$^{-/-}$ mice [area: one-way ANOVA, $F_{(3,\ 305)} = 51.36$, $p \leq 0.0001$, *snk post-hoc* test CF$^{+/+}$ vs. CF$^{-/-}$ significantly different, $p \leq 0.0001$; circularity index: one-way ANOVA, $F_{(3,\ 302)} = 8.178$, $p \leq 0.0001$, *snk post-hoc* test CF$^{+/+}$ vs. CF$^{-/-}$ significantly different, $p \leq 0.05$]. When WT mice were challenged with a MHF diet, the mitochondrial cross-sectional area but not the circularity index became enlarged [area: one-way ANOVA, $F_{(3,\ 305)} = 51.36$, $p \leq 0.0001$, *snk post-hoc* test CF$^{+/+}$ vs. MHF$^{+/+}$ significantly different, $p \leq 0.0001$; circularity index: one-way ANOVA, $F_{(3,\ 302)} = 8.178$, *snk post-hoc* test CF$^{+/+}$ vs. MHF$^{+/+}$, $p \geq 0.05$] and the abundance of mitochondria decreased [number: $F_{(3,161)} = 4.925$, $p = 0.0027$, *snk post-hoc* test CF$^{+/+}$ vs. MHF$^{+/+}$ significantly different, $p \leq 0.05$]. In contrast, a MHF-diet challenge did not affect mitochondria number, area, or circularity in the Kv1.3$^{-/-}$ mice (one-way ANOVA, *snk post-hoc* test, $p \geq 0.05$). These data provide evidence that diet high in fat changes the morphology of mitochondria in the OB, and that gene-targeted deletion of Kv1.3 precludes these effects.

DISCUSSION

The voltage-gated *Shaker* potassium channel Kv1.3 is a key contributor in regulating body weight, metabolism, and energy homeostasis. Fewer data are reported elucidating specific potential molecular mechanisms linking Kv1.3 kinetics and energy balance. Herein we provide new evidence for co-localization of Kv1.3 and the glucose transporter type 4 (GLUT4) in the mitral cell layer of the OB. We further demonstrate that GLUT4 is a possible substrate for Kv1.3 activation whereby functional Kv1.3 increases GLUT4 expression on the membrane and Kv1.3 current suppression strongly upregulates GLUT4 transport from intracellular storage to the plasma membrane. Additionally, we show that gene-targeted deletion of Kv1.3 channel changes mitochondrial shape and reduces their size. Moreover, WT mice that are challenged with a MHF-diet exhibit a decrease in number of mitochondria within the mitral cells of the OB whereas those from Kv1.3$^{-/-}$ mice do not demonstrate such loss. Mitochondria within Kv1.3$^{-/-}$ mice are also resistant to increase in size following MHF-challenge, which is observed in WT mice.

As summarized in **Table 1**, GLUT4 is expressed in several discrete brain regions such as the hypothalamus, the hippocampus, the cerebellum, and the amygdala. GLUT4 in the brain is mainly neuronal, and localizes on dendrites or on the membranes of transport vesicles, Golgi apparatus, and the rough endoplasmic reticulum (Kobayashi et al., 1996; Leloup et al., 1996; McCall et al., 1997; El et al., 1998; Choeiri et al., 2002). In the OB, GLUT4 proteins are localized on the dendritic terminals of mitral/tufted cells at the level of glomeruli, which are sites of synaptic connectivity between the axons of OSNs and mitral cells, and in the nuclei of periglomerular cells (Sharp et al., 1975). Our *in situ* hybridization results indicate that GLUT4 and Kv1.3 transcription occurs in the cell bodies of mitral cells and interneurons before being translated and recruited at the synapse for glucose-based modulation of olfactory information. Interestingly, Sharp and collaborators also found that GLUT4 protein was co-localized with IR on mitral cells (Sharp et al., 1975, 1977). This is highly consistent with our characterization of IR modulation of Kv1.3 via tyrosine phosphorylation on the N- and C-terminal aspects of the channel, and our finding of co-localization of the channel with the kinase in mitral cells (Fadool et al., 2000). Because our previous electrophysiological data demonstrate nearly all mitral cells are insulin sensitive (Fadool et al., 2011), combined with our current gene expression data and the protein data from the Sharp laboratory (Sharp et al., 1975, 1977), it is highly plausible that all three signaling molecules are contained within mitral cells (**Figure 4**). Although it remains to be shown if all mitral cells are homogeneous in their expression of Kv1.3/GLUT4/IR or whether there is a degree of heterogeneity across this population of first order neurons (Angelo et al., 2012; Padmanabhan and Urban, 2014), the modeled signaling interactions could influence glucose sensing, and concurrently, electrical signaling, by mitral cells. Decreased Kv1.3 activity evokes translocation of GLUT4 to the membrane to permit glucose uptake and the end production of ATP. In our model, we demonstrate two known modulators of

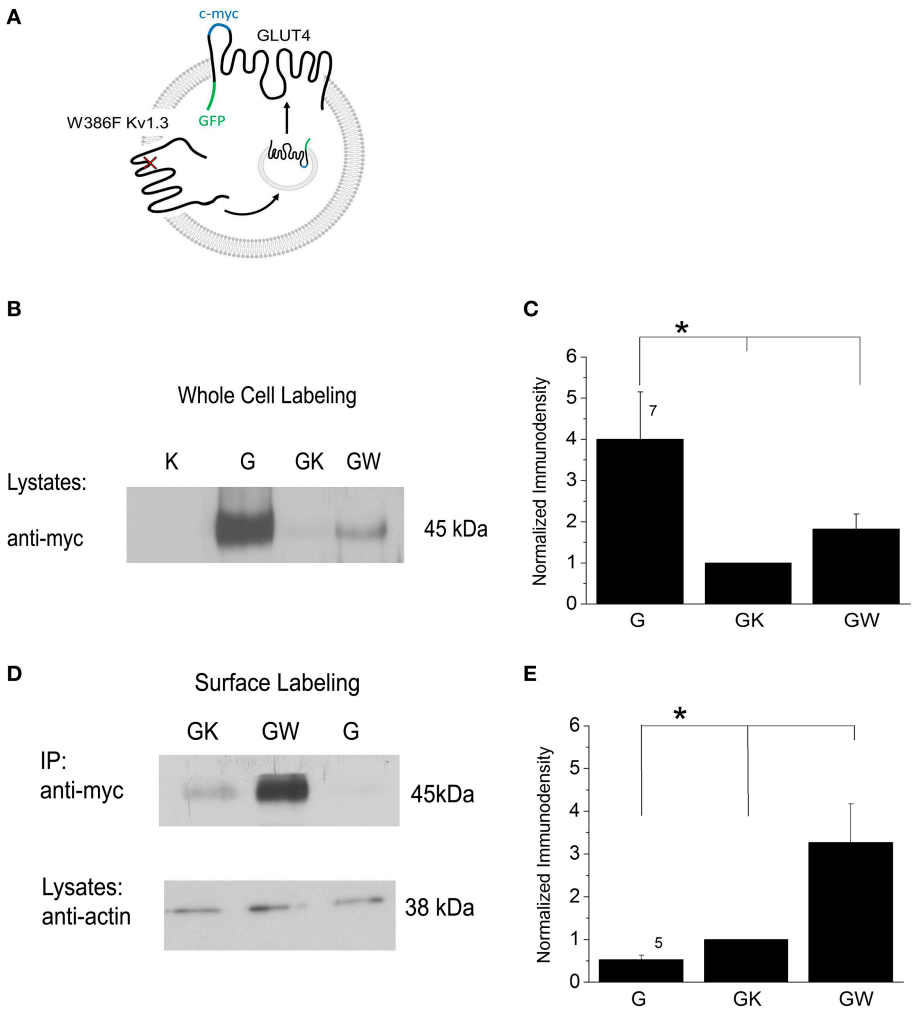

FIGURE 2 | GLUT4 is expressed intracellularly and then translocates to the plasma membrane following block of Kv1.3 channel achieved by site-directed mutagenesis of the pore. (A) Model of heterologous co-expression of a non-conducting Kv1.3 channel (W386F Kv1.3) and an extracellular epitope-myc tagged GLUT4 transporter that becomes translocated to the plasma membrane. **(B)** Western blot of lysates harvested from HEK293 cells transfected with K: Kv1.3 cDNA; W: mutant W386F Kv1.3 cDNA; G: GLUT4-myc cDNA. Blots were probed with myc antibody (anti-myc) to visualize labeling of all GLUT4 in a whole-cell preparation. **(C)** Bar graph of the normalized pixel immunodensity of the quantified bands for 7 such blots as represented in **(B)**. * = one-way analysis of variance (ANOVA), Bonferoni *post-hoc* test, $p \leq 0.05$. Different length vertical lines represent significantly different *post-hoc* comparison. **(D,E)** Same as **(B,C)** but samples were surface immunoprecipitated (IP) with myc antibody (anti-myc) and then labeled with anti-myc to visualize labeling of surface GLUT4. Input (lysates) were labeled with actin antibody (anti-actin) to standardize equal loading.

Kv1.3 activity that are related to incretin hormones (glycogen-like peptide signaling and IR kinase), which decrease Kv1.3 conductance through either a confirmed (Fadool and Levitan, 1998; Fadool et al., 2000, 2011) or suspected (Thiebaud et al., 2016) phosphorylation (P) of the channel. Our mutagenesis data directly demonstrate that blocked conduction of Kv1.3 current is solely capable of GLUT4 translocation and this is consistent with earlier pharmacological experiments from the Desir laboratory that applied known potassium channel blockers to visualize surface expression of GLUT4 by immunocytochemistry (Xu et al., 2004). Because our HEK cell model expresses only a few components of the IR signaling cascade (Thomas and Smart, 2005) and our experiments were performed without the hormone insulin, our biochemical data demonstrate the ability of Kv1.3 and GLUT4 to interact without the necessity of insulin to either translocate GLUT4 or alternatively to phosphorylate Kv1.3. We conjecture that in native mitral cells, insulin could induce GLUT4 translocation either through direct IR activation or through Kv1.3 inhibition caused by IR phosphorylation (blue and red dashed lines, respectively; **Figure 4**). Our model could include downstream signaling well characterized in the periphery, however, the existence of these parallels in the CNS is not known. For example, Kv1.3 inhibition enhances GLUT4 translocation in adipocytes through a PI3K-independent mechanism consisting of depolarizing the plasma membrane and increasing intracellular Ca^{2+} concentration (Li et al., 2006).

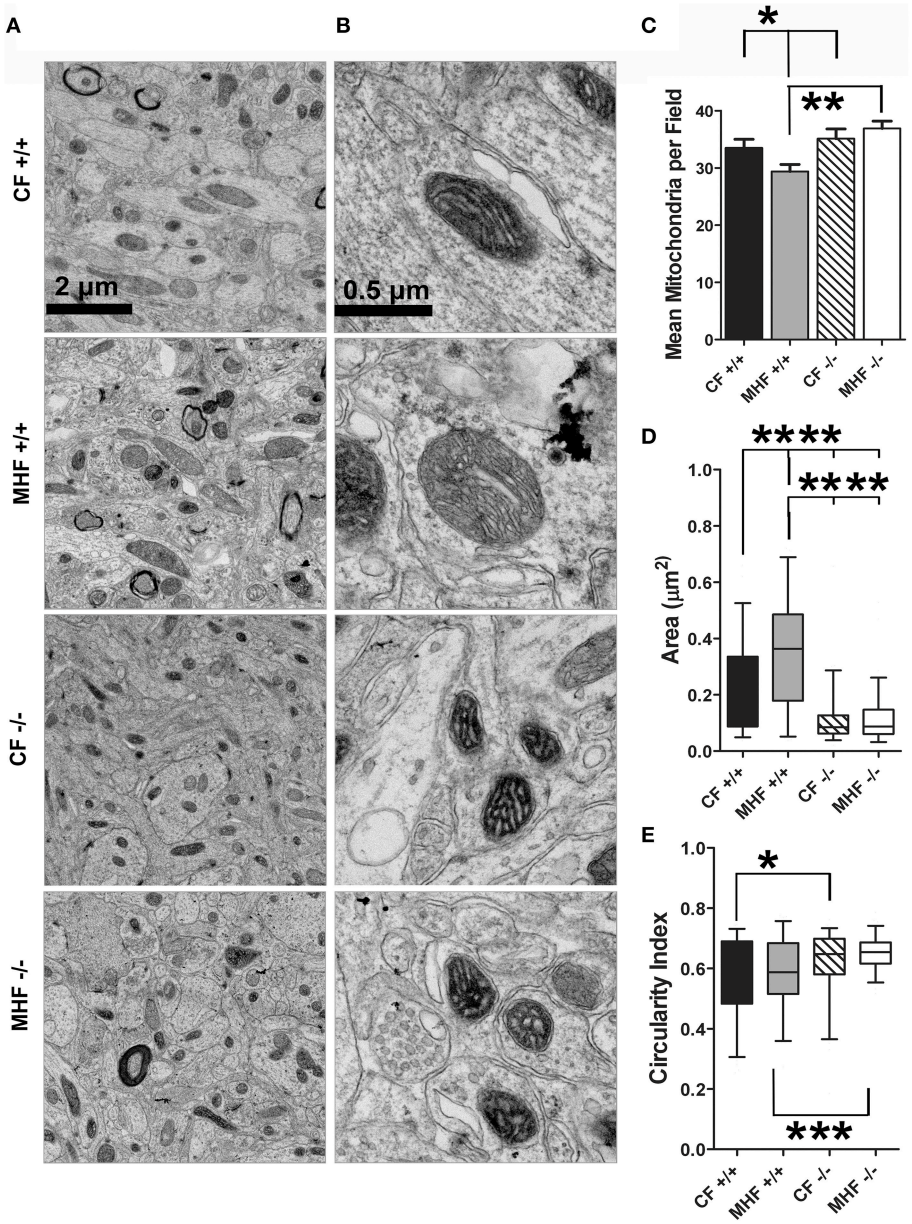

FIGURE 3 | Kv1.3$^{-/-}$ mice have smaller mitochondria in the mitral cell layer of the OB, which do not exhibit an increase in volume when challenged with moderately-high fat diet (MHF). Photomicrographs acquired from the mitral cell layer of the OB of wildtype (+/+) and Kv1.3-null mice (–/–) maintained on MHF vs. CF for 4 months. **(A)** Low and **(B)** higher magnification. Bar graph or box blot representing **(C)** mean number, **(D)** cross-sectional area, and **(E)** circularity index of mitochondria collected across 40 fields of view for 3 mice. Data represent mean +/− s.d. for the bar graph, or mean (line), 25/75% quartile (box) and 5/95% (whiskers) for the box plot. Significantly different, one-way ANOVA, Bonferoni's *post-hoc* test, $^{****}p \leq 0.0001$; $^{***}p \leq 0.001$, $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

GLUT4 translocation to the membrane is classically facilitated by insulin binding to its receptor, which triggers PI3K-dependent or -independent pathways (Hoffman and Elmendorf, 2011). Another interesting caveat is that GLUT4 expression in the OB seems to be modulated by the feeding state. Postprandially when glucose levels in the blood are high, GLUT4 is expressed on the plasma membrane of dendritic processes. GLUT4 in fasted rats however, is internalized into the cytoplasm (Al Koborssy et al., 2014) even though insulin levels in the OB are shown to be 15 fold higher in the fasting state (Fadool et al., 2000). Our biochemical data point to a role of Kv1.3 in controlling GLUT4 expression on the membrane but more evidence is needed to prove that this modulation could bypass IR kinase *in vivo*.

Because Kv1.3$^{-/-}$ mice have an increased locomotor activity and metabolism, a change in mitochondrial shape might be anticipated. Our ultrastructural data demonstrate that control fed Kv1.3$^{-/-}$ mice have an altered circularity shape compared with similarly fed WT mice. Additionally, the cross-sectional area of

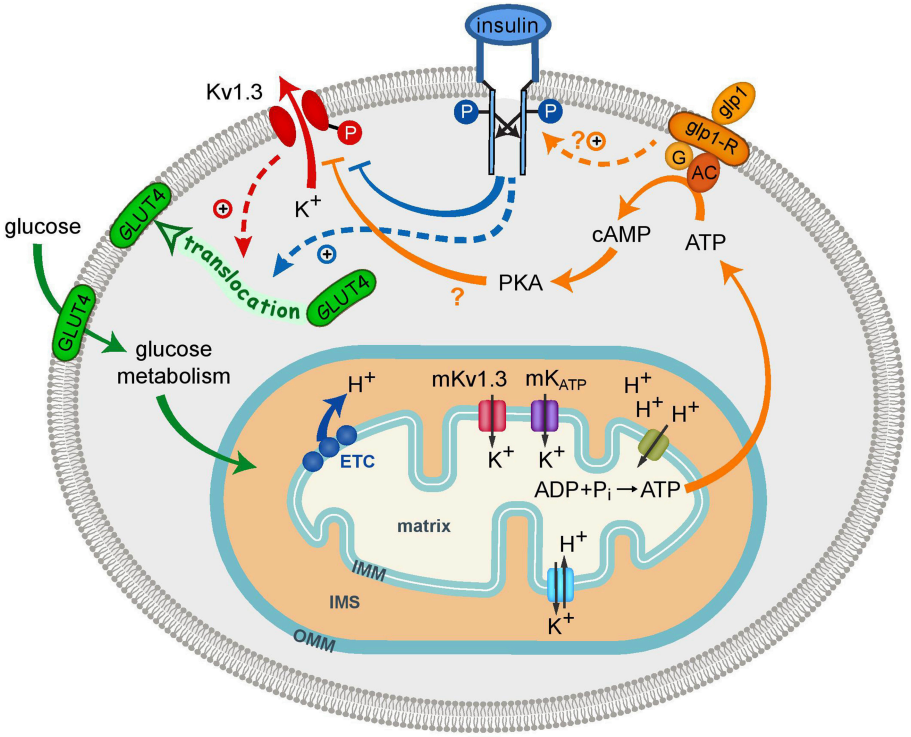

FIGURE 4 | Schematic diagram showing cell signaling interactions of plasma membrane Kv1.3 (Kv1.3) and potential interplay with mitochondrial Kv1.3 (mKv1.3). Glycogen-like peptide (GLP-1) and insulin are two signaling hormones that block Kv1.3 current. The channel (red) is known to be a substrate for insulin receptor (IR) kinase (blue) on the N- and C-terminal aspects of the channel protein (Y111-113, Y137, Y479). Insulin-induced phosphorylation (P) of Kv1.3 (blue line) decreases Kv1.3 current amplitude by decreasing Pr_{open} of the channel. GLUT4 is typically translocated to the membrane upon insulin activation (blue dashed line) but upon block of Kv1.3 conductance, can also be translocated (red dashed line). Metabolism of glucose establishes the H^+ gradient and the production of ATP via the electron transport chain (ETC). Maintenance of the ionic environment is dependent upon influx of K^+ through mKv1.3 and mKATP and the balance provided by efflux via the K^+/H^+ exchanger. The ATP energy source could be used for the GLP-1R triggered conversion of ATP to cAMP through adenylase cyclase to increase potential PKA activity (?). Metabolic factors that decrease Kv1.3 ion channel activity increase the AP firing frequency in mitral cells that is thought to provide odor quality coding of olfactory information. OMM, outer mitochondria membrane; IMM, inner mitochondria membrane; IMS, inter membrane space.

the mitochondria is smaller in the Kv1.3$^{-/-}$ regardless of dietary treatment. Neuronal mitochondria typically have cristae that form a network of anastomosing tubes and the number of cristae is correlated with the level of aerobic activity and associated ATP production (Lehninger, 1982). While our analysis did not quantify this sub-organelle structure, qualitatively the cristae from the WT mice appeared to be more longitudinally arranged. During high rates of metabolism, the mitochondrial membranes can become condensed due to structural changes of the IMM and matrix, as opposed to orthodox or "idling" mitochondria that are not actively respiring (Lehninger, 1982). This type of shrinking does not appear to be the cause of smaller mitochondria in the Kv1.3$^{-/-}$ because the outer mitochondrial membrane (OMM) is observed closely juxtaposed to the IMM and cristae. Future functional studying of mitochondria from Kv1.3$^{-/-}$ mice are needed to test the idea that the more condensed mitochondrial volume might contribute to increased TEE. Interestingly in the rat OB, the respiratory control ratio that measures mitochondrial activity is 2x higher that in the hypothalamus (Benani et al., 2009) and is in accordance with the high energy demand of the glomeruli (Nawroth et al., 2007). What is also of interest for mitochondria in the OB is the high expression of GLUT4 in this region and the total energy budget for olfactory discrimination. Odor-evoked oxidative metabolism of OB synaptic transmission is energetically demanding and is tightly correlated to capillary density (Lecoq et al., 2009).

The challenge of a MHF-diet did not decrease the number or increase cross-sectional area of mitochondria in the Kv1.3$^{-/-}$ mice as it did in the WT mice. This is very intriguing given that mitochondria are highly dynamic in morphology and abundance. In liver, short-term challenge (21 day) with a high-fat diet causes a decrease in proteins of the electron transport chain and transporters/exchangers of the IMM, but does not cause a change in mitochondrial volume (Kahle et al., 2015). The influx of potassium across the IMM (**Figure 4**) through both mKv1.3 and mK_{ATP} could cause an increase in volume changes of the mitochondria if not tightly counterbalanced by the H^+/K^+ antiport exchanger (Garlid, 1996). One role of the H^+/K^+ antiport exchanger is to counterbalance the influx of K^+ that allows the high membrane gradient required for oxidative phosphorylation and thereby acts to provide volume homeostasis (Garlid and Paucek, 2003). In the absence of Kv1.3, there may be

less K^+ influx, leading to smaller mitochondria. In the condition of long-term (4 months) MHF-diet and precipitated DIO, the WT mice could have dysfunction in IMM ion transport resulting in increased volume or swelling. Such ionic imbalance could lead to unhealthy mitochondria, which might undergo mitophagy and lead to a loss in mitochondrial density or abundance such as we observed. The subsequent lack of volume change or loss of number in the fat-fed Kv1.3$^{-/-}$ mice could be either attributed to less initial K^+ influx or changed fatty-acid metabolism that is also linked to organelle volume (Garlid, 1996) and would be anticipated to be reduced in the thin, obesity resistant mice.

Whether mitochondrial dysfunction is linked to glucose intolerance and insulin resistance is a topic of intensive research and current debate (see review, Montgomery and Turner, 2015) but certainly a greater number of mitochondria permit greater ATP production and a reduced number of mitochondria are found during periods of quiescence. Typical to what we observed in the DIO wildtype mice, a reduced number of mitochondria was associated with larger individual organelles. Although our ultrastructural data do not allow us to discern if the larger mitochondria had impaired function, by deduction, the fatty diet either decreased mitochondrial biogenesis or increased mitophagy to yield a decreased mitochondrial density in the obese mice. The fact that loss of Kv1.3 channel alters mitochondria shape and prevents increased volume of mitochondria when challenged with MHF suggests an alteration of normal dynamics of the organelle and associated fission and fusion proteins. Similar effects of high-fat diet on mitochondrial function have been observed in the central nervous system (Petrov et al., 2015). For example, Parton et al. (2007) attributed loss of glucose sensing by pro-opiomelanocortin (POMC) neurons of the hypothalamus in DIO mice to the mitochondrial uncoupling protein 2 (UCP2). Genetic deletion or pharmacological inhibition of UCP2 was able to restore glucose sensing in the obese mice, underscoring the role of mitochondrial dysfunction and altered ATP production in DIO.

One of the earliest changes in the development of insulin resistance attributed to DIO is ectopic lipid accumulation (Turner et al., 2013). In response to excess lipid availability in the wildtype DIO mice, increase reactive oxygen species (ROS) such as superoxides, hydroxyl radicals, and hydrogen peroxide could have been elevated (i.e., Paglialunga et al., 2015). Interestingly, Kv1.3 can be regulated by these types of second messengers. Phosphorylation of Kv1.3 on four serine residues by PKA and PKC is known to increase Kv1.3 activity to maintain a negative membrane potential (Chung and Schlichter, 1997a,b). As ATP is depleted below basal intracellular levels, the channel exhibits reduced peak current amplitude and a shift in voltage dependence. Application of ROS scavengers alleviates Kv1.3 phosphorylation to decrease the membrane potential (Cayabyab et al., 2000).

Plasma membrane bound Kv1.3 and mitochondrial Kv1.3 could influence or detect metabolic state at the level of the OB through channel neuromodulation and down-stream signaling cascades that could modify excitability. Insulin and the incretin hormone, GLP-1, can phosphorylate Kv1.3 at the plasma membrane, decrease Kv1.3 current and upregulate GLUT4 translocation to the membrane to facilitate glucose metabolism. In turn, the activity of mitochondrial Kv1.3 is linked to ROS and ATP production that regulate channel phosphorylation and conductance. Future targeted regulation of Kv1.3 in the OB could reveal the ability of this brain region to detect and regulate metabolic state by changing membrane excitability based on nutritional needs. The extent by which the mitral cell plasma and mitochondrial membrane activity interplays in detecting the chemistry of metabolism and olfactory coding utilizing Kv1.3 is a novel future trajectory in understanding brain energy sensing and dysfunction following obesity.

AUTHOR CONTRIBUTIONS

DF conceived of the experiments; JF and DK conducted the *in situ* hybridization experiments; KK and JF conducted the electron microscopy (EM) experiments; DF, BC, and KK conducted the biochemistry experiments; DK and ZH analyzed the biochemistry and EM results respectively; all authors drafted the manuscript and critically evaluated the final content.

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Odor-Induced Neuronal Rhythms in the Olfactory Bulb Are Profoundly Modified in ob/ob Obese Mice

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Leptin, the product of the Ob(Lep) gene, is a peptide hormone that plays a major role in maintaining the balance between food intake and energy expenditure. In the brain, leptin receptors are expressed by hypothalamic cells but also in the olfactory bulb, the first central structure coding for odors, suggesting a precise function of this hormone in odor-evoked activities. Although olfaction plays a key role in feeding behavior, the ability of the olfactory bulb to integrate the energy-related signal leptin is still missing. Therefore, we studied the fate of odor-induced activity in the olfactory bulb in the genetic context of leptin deficiency using the obese ob/ob mice. By means of an odor discrimination task with concomitant local field potential recordings, we showed that ob/ob mice perform better than wild-type (WT) mice in the early stage of the task. This behavioral gain of function was associated in parallel with profound changes in neuronal oscillations in the olfactory bulb. The distribution of the peaks in the gamma frequency range was shifted toward higher frequencies in ob/ob mice compared to WT mice before learning. More notably, beta oscillatory activity, which has been shown previously to be correlated with olfactory discrimination learning, was longer and stronger in expert ob/ob mice after learning. Since oscillations in the olfactory bulb emerge from mitral to granule cell interactions, our results suggest that cellular dynamics in the olfactory bulb are deeply modified in ob/ob mice in the context of olfactory learning.

Keywords: leptin, ob/ob mice, odor-induced oscillations, learning, olfactory bulb

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INTRODUCTION

Dysregulation in the control of food intake is a major contributor to the rising number of obese people and strongly contributes to the obesity epidemic related lethal complications such as cardiovascular diseases and type 2 diabetes (Dietrich and Horvath, 2012). A fine tuning of feeding behavior is made possible by the central integration of peripheral energy-related circulating signals released at the periphery of the brain (Luquet and Magnan, 2009). Among these hormones, leptin is a known anorectic signal mainly expressed by the adipose tissue according to energy store (Zhang et al., 1994; Friedman and Halaas, 1998). Increasing plasma level of leptin in time of energy abundance leads to decrease in food intake and increase energy expenditure, mostly through action of leptin on hypothalamic neural substrate (Prieur et al., 2008). Disruptions in any component

of the leptin signaling pathway invariably leads to hyperphagia, obesity, and corollary disease in both human and rodents (Schwartz and Porte, 2005). Likewise, the leptin-null ob/ob mice exhibit decreased energy expenditure, hyperphagia and obesity (Lindström, 2007). In addition, the obesity state *per se* is associated with the ability for leptin to properly access and regulates central neural substrate and is refereed as to "leptin resistance." "Leptin resistance" is characterized with a state of reduced responsiveness to plasma leptin, hampering the inhibition of food intake (Zhang et al., 1994; Lee et al., 2001; Farooqi, 2006). The primary target of leptin binding in the brain is the hypothalamus. However, it was observed that neurons elsewhere in the brain (Hayes et al., 2010), including the olfactory bulb (OB), the first location in the brain to perform a spatiotemporal coding of odors, also express significant amounts of leptin receptors (Shioda et al., 1998; Prud'homme et al., 2009).

Olfactory cues are determinant for a variety of behaviors including social interactions and, importantly, for feeding behavior (Rolls, 2005; Yeomans, 2006). The OB is modulated by numerous hormones and nutrients involved in metabolic regulation (Palouzier-Paulignan et al., 2012). Both leptin receptor mRNA and immunoreactivity were found in the forebrain, in regions that massively project onto the OB, the piriform cortex and the entorhinal cortex (Elmquist et al., 1998; Shioda et al., 1998; Ur and Wilkinson, 2001) and also peripherally, in the olfactory mucosa (Baly et al., 2007). Within the OB, labeling of both long and short isoforms of the leptin receptor was observed in numerous mitral cells, in granule cells as well as on astrocytes in the glomerular and granule cells layers (Prud'homme et al., 2009). Leptin receptor immunoreactivity was found in the internal granular layer, one of the layers receiving the feedback projections. These data suggest a role for leptin in olfactory function. Indeed, in humans, a correlation has been observed between leptinemia and odor pleasantness (Trellakis et al., 2011) or odor identification (Karlsson et al., 2002). Leptin was shown to be a potent regulator of olfactory receptor neurons activity by increasing spontaneous activity and decreasing odor-evoked activity (Savigner et al., 2009). Central administration of leptin in fasted rats induces a dose-dependent decrease of olfactory detection (Julliard et al., 2007) and reduces food-odor exploration (Prud'homme et al., 2009). Accordingly, ob/ob mice detected a cracker hidden under the cage bedding faster than their wild-type littermates (Getchell et al., 2006): thus the lack of leptin seems to induce a surprising gain in olfactory function. Taken together, these data suggest that leptin influences olfactory abilities although precise mechanisms are still unknown.

Odorant molecules are detected by the olfactory receptor neurons located in the olfactory mucosa, on the caudal part of the nasal cavity (Mombaerts, 1999). Each olfactory receptor neuron (ORN) expresses one of the several hundreds of olfactory receptors (around a thousand in rodents, one third of this number in Humans) and projects to the OB. Within the olfactory glomeruli located at the surface of the OB, ORN makes excitatory synapses with the main cells of the OB, the mitral cells (Pinching and Powell, 1971). Two populations of inhibitory interneurons modulate the olfactory signal processing within the OB, the periglomerular cells located at the superficial layers and the granule cells present in deeper layers (Nagayama et al., 2014). Mitral cells project directly to neurons in the olfactory cortex (Mainland et al., 2014), mainly to the piriform cortex, which send centrifugal feedback (CF) to the OB. Olfactory cortical feedback activity tightly regulates OB interneurons activity (Boyd et al., 2012, 2015).

The olfactory system is recognized as a highly oscillatory system. Odor processing in freely moving rodents has been associated with specific modulations of the OB and the piriform cortex local field potential oscillations (LFP) (Kay, 2014; Martin and Ravel, 2014). These oscillations are also modulated by the metabolic state: oscillations elicited by food odors are enhanced in food deprived rat (Chabaud et al., 2000). Most of the time, in the context of appetitive learning, odorant sampling elicits high power oscillations in the beta (15–40 Hz) frequency band (Martin et al., 2004; Fuentes et al., 2008; Lepousez and Lledo, 2013; Chery et al., 2014). Because they underlie coincident activity, oscillations would favor temporal coordination of sensory information within brain areas and facilitation of its transfer across regions (Varela et al., 2001; Siegel et al., 2012). They are also ideally suited to subserve memory processes such as encoding, consolidation and retrieval (Engel et al., 2001; Fell and Axmacher, 2011). In addition, abnormalities of rhythmic activities in neural dynamics provide a reliable readout of neural circuit status (Cramer et al., 2012; Uhlhaas and Singer, 2015).

To date, there were no studies investigating the plasticity of olfactory behavior and related temporal dynamics in the olfactory network in the absence of leptin. We used LFP recordings in the ob/ob obese mice to study oscillatory dynamics in the OB linked to performance in an odor discrimination task. We found significant changes of neuronal oscillations in the gamma and above all in the beta frequency range suggesting that the lack of leptin induces profound changes in the interaction between the mitral and the granule cells under the influence of centrifugal inputs from the olfactory cortex.

MATERIALS AND METHODS

Animals

All protocols were approved by the Institutional Animal Care and Use Committee (Comité d'Ethique en Expérimentation Animale Paris Centre et Sud n°59) with the ethical agreement 01847.01 and carried out in compliance with EU legislation (Directive 2010/63/EU).

C57Bl/6j and ob/ob male mice were purchased from Janvier Labs (Le Genest-St-Isle, France) at the age of 6 weeks and fed with a regular diet for adult mice (A04 Diet, Safe, France). They were kept at 22 ± 0.5°C, 50 ± 5% humidity, and maintained on a 12 h light dark cycle. Animals were housed in group until the surgical procedure, and housed individually afterward. Food and water were available *ad libitum* except during the behavioral procedure when in addition to the sucrose solution received in the experimental chamber, access to water was restricted and given once a day (1 ml per mouse in their home cage at 05:00 p.m. in addition to the 0.4 mL sucrose received in the experimental chamber). Weight was measured every day during the behavioral test.

To avoid the stress of the puncture to animals involved in the learning experiment, we used a group of mice paired in age and weight to monitor glycemia. Measures were performed in ad lib condition or after 17 h-overnight fasting, by collecting a drop of blood in the tail of each mouse and using a Glucofix Monitor (Menarini Diagnotics, France).

Electrodes Implantation

Mice were implanted with a single electrode in the OB. Anesthesia was induced by 1.5–2% isoflurane, and reduced to 0.5–1% once the mouse was asleep. The level of anesthesia was confirmed by toe pinch and the absence of ocular reflex. The electrode (diameter 125 μm, stainless steel, Plastic One) was positioned stereotaxically (4.5 mm anterior, 1 mm lateral and 0.8–1.1 mm ventral relative to Bregma) at the level of the granule cells layer using electrophysiological monitoring of the signal characteristics (gamma bursts and respiratory modulation). The reference electrode was connected to a skull screw located above the posterior portion of the contralateral cortical hemisphere. The connector of the electrode was fixed on the top of the mouse's head with dental acrylic cement. A recovery period of 2 weeks followed surgical procedure before behavioral experiment.

Electrophysiological Recordings and Odorant Delivery

LFP signals were recorded during the entire behavioral training. Mice were connected to the recording device by a tether plugged into the implanted connector. Monopolar activity was acquired using a custom DasyLab (IOTECH, U.S.A) script driving an XCellAmp 64 amplifier (Dipsi, France) coupled with a DaqBoard 3000 USB system (IOTECH, U.S.A). Signal was sampled at 2000 Hz, amplified (x 2500) and digital filters were set at 0.1–300 Hz. Odorants were delivered using an automated perfusion system (ValveBank II, AutoMate Scientific, U.S.A) that controlled the duration and the flow rate of the stimulation (odorized air flow). A piece of filter paper, loaded with 50 μL of the odorant was used to odorize the flow. All the experiments took place in a grounded Faraday cage.

Operant Behavior

Mice were trained in a Go/No-Go task based on odor discrimination (cf. **Figure 1A**). The experimental cage (Habitest, Coulbourn Instruments, U.S.A) was a box containing one wall equipped with two ports: (1) a self-delivery drinking system consisting of a liquid dipper and (2) a separated odor port connected to the olfactometer. Both ports were equipped with beam detectors. Clean air constantly flowed through the stimulation port. Detection of a mouse nose poke in this port triggered odorant delivery for 2 s. After odor sampling, a nose poke in the liquid dipper triggered distribution of 20 μL of sweetened water (6% sucrose in water) which remained accessible for 10 s. The whole system was controlled by Graphic State (Coulbourn Instruments, U.S.A.). The behavioral experiment was preceded by a 24 h water restriction, then the standard restriction (1 ml/mouse/day) started.

During the shaping phase, every nose poke in the odor port automatically initiated odorant delivery and triggered reward distribution. Shaping ended when the animals performed at least 20 nose pokes in one session. During the test sessions, nose poke in the odor port initiated the delivery of the odorant. Two odorants were pseudorandomly delivered for the same amount of trials per session. When animals received the odorant corresponding to Go trials they had to nose poke in the dipper to receive the reward. When it was the odorant corresponding to No-Go trials, no reward was delivered, nose poke in the dipper initiated a penalty intertrial interval of 20 s. A trial was set to 6 s including odorant delivery. A correct response was when the animal nose-poke in the dipper before the end of the trial for Go trials and when they did not nose-poke in the dipper before the end of the trial for No-Go trials. A session ended after 40 trials.

The odorant used for the shaping phase was eugenol (Sigma Aldrich, Saint-Quentin Fallavier, France) diluted at 10% in mineral oil, for the test phase, hexanal (Sigma Aldrich) diluted at 10% in mineral oil was used for Go trials, butanal (Sigma Aldrich) diluted at 15% in mineral oil was used for No-Go trials.

Data Analysis

Behavior

All the behavioral parameters were automatically recorded. For every trial performed by the animals, we calculated the latency between the odor onset (nose poke into the odor port) and the nose poke into the liquid dipper. For the day to day analysis, values were averaged by block of 10 consecutive trials for each odor. The percentage of correct trials was calculated for every session. Criterion performance was set at 2 consecutive days of 90% correct choice in total, including at least 80% correct choice for the No-Go trials.

LFP Analysis

All electrophysiological data were exported and stored in a MySQL database for subsequent analysis. Data were processed using OpenElectrophy software (http://packages.python.org/OpenElectrophy; Garcia and Fourcaud-Trocmé, 2009). Data were visually inspected individually to discard trials containing artifacts.

Because the signal recorded in the OB is highly modulated in time and characterized by transient changes in frequency, we chose to use wavelet analysis (Mallat and Peyré, 2009), a particularly powerful tool for studying transient phenomena without any prior knowledge of frequency bands of interest (Tallon-Baudry and Bertrand, 1999). A continuous Morlet wavelet transform was then applied between 5 and 160 Hz and between 3 s before odor onset to 3 s after odor onset, resulting in an estimate of oscillatory power for each time and frequency values. We obtained time frequency matrix where each point represents the level of energy for a given time and a given frequency: hot color spots represent transitory oscillations.

For each map corresponding to one trial, we focused our analysis on a 1 s baseline period, before odor onset (from 2 to 1 s prior to odor onset) and a 1 s odor period, during odorant stimulation (from 0 to 1 s after odor onset). We conducted separated detection analysis for two frequency band of interest: beta (15–40 Hz) and gamma (60–150 Hz).

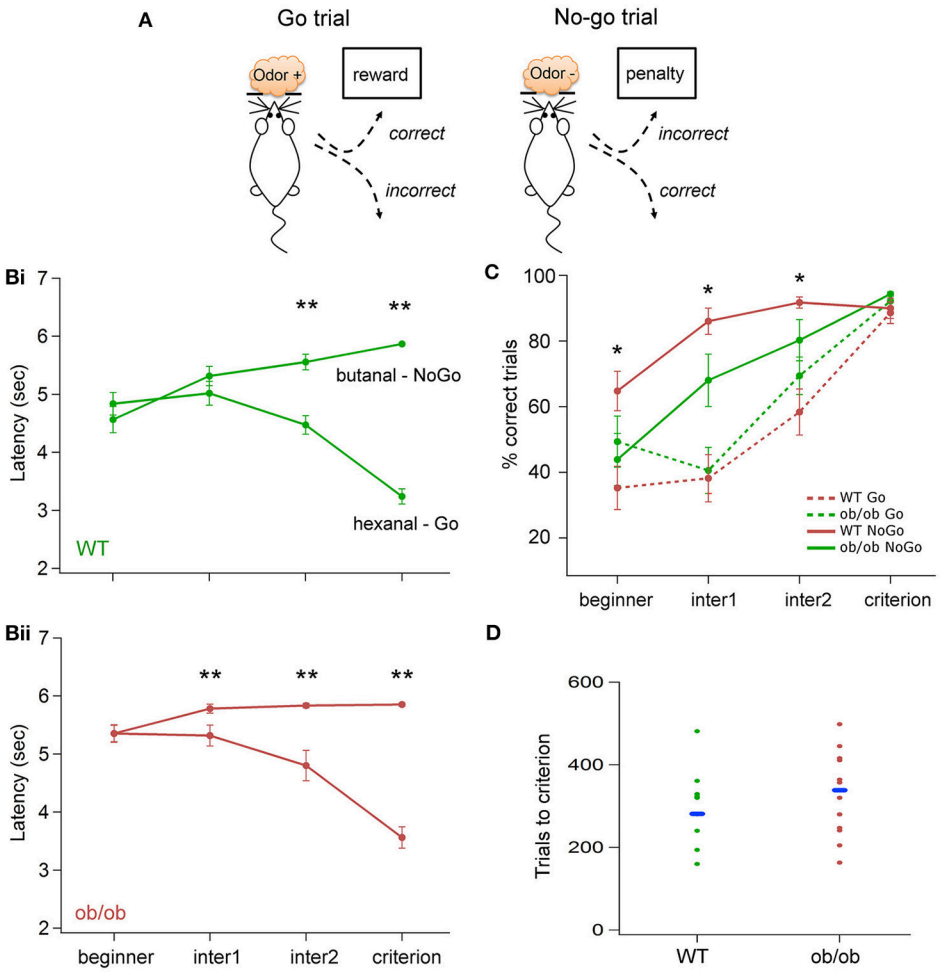

FIGURE 1 | Odor discrimination performances in WT and ob/ob mice. (A) Schematic picturing the behavioral paradigm of the Go/No-Go task. According to the odor sampled in the odor port, a reward or a penalty was received if the animal poked into the dipper (see Materials and Methods section for details). Fifty percentage of each type of trials were delivered pseudorandomly. **(B)** Evolution of the latencies during the training for WT **(Bi)** and ob/ob **(Bii)** mice. Latency was measured for every trial as the duration between odor onset and nose poke in the dipper (correct No-Go was assigned the maximum latency of 6 s). The test was divided in 4 periods of time: *beginner,* the first 2 sessions of the Go/No-Go test, *criterion,* the 2 sessions at which mice were at the set criterion. The sessions in between were equally divided in two: *inter1* and *inter2* (see the text for details). Each value represented the average of sessions of the corresponding phase for all animals in each group (Bi: WT $n = 14$; Bii: ob/ob $n = 10$). In the ob/ob group, latency started to be significantly different for Go and No-Go trials for *inter1*, whereas significance was reached in *inter2* for the WT group (**$p < 0.01$ Mann-Whitney test). **(C)** Evolution of the percentage of correct responses for the Go and No-Go stimulations in the two groups. Performances are not significantly different between ob/ob and control mice for the Go trials but ob/ob mice have significantly better performances for the No-Go trials (*$p < 0.05$ Mann-Whitney test). **(D)** Number of trials performed by mice to reach the criterion level in the two groups. Each dot denotes one mouse. Dashes indicate the median for each group. The number of trials necessary for the mice to reach the criterion was not significantly different between ob/ob and WT animals.

We first calculated LFP mean power during the baseline and odor periods in the two frequency band. We then extracted features of oscillations bursts using a method based on the wavelet ridge extraction previously described by Roux et al. (2007) and applied to biological signals by Cenier et al. (2008). Briefly, it consisted in detecting local maximum energy points above a threshold to compute the wavelet ridge, i.e., the path of lowest energy decrease. The instantaneous frequency and phase of the signal were then extracted from the ridge. To ensure that we analyze oscillations, only the bursts presenting at least 3 cycles were kept for subsequent analysis. For each trial, we extracted the number of bursts above threshold, their energy, frequency, temporal position, onset and duration.

Considering the characteristics of oscillatory activity in the OB as previously detailed (Martin and Ravel, 2014), for beta band analysis, in each trial, bursts were extracted during the odor period, with a threshold calculated at +3STD of the average value of the baseline in the same frequency range. Since gamma activity decreased during odor sampling, bursts were analyzed from the baseline with a threshold calculated from the same region of interest (baseline, 60–150 Hz). Then, the threshold was set at +2STD of the average value of the baseline to ensure the extraction of all gamma bursts.

Histology

Following the end of all experiments, the animals were sacrificed by a lethal dose of pentobarbital and an electrocoagulation (5 repetitions, 10 mA, 3 s) was performed through the electrode. Brain was removed and frozen. 40 μm thick coronal slices were sectioned with a cryomicrotome and Nissl staining was performed for subsequent histological examination of electrode location.

Statistical Analysis

All tests were performed comparing leptin (ob/ob) mutant mice to age- and gender-matched wild-type (WT) mice. Statistical analyses were performed using nonparametric tests. Effects of the following factors were tested: on the power, frequency and duration values, and on the number of bursts above threshold. Three independent factors were tested, the group (WT vs. ob/ob), learning (beginner vs. criterion) and the odorant (hexanal vs. butanal) using Mann-Whitney *U*-tests. One paired factor, the period factor (baseline, odor), was tested using Wilcoxon paired-samples tests. X^2-test was used to test for differences in repartitions between the two groups.

RESULTS

We challenged ob/ob mice ($n = 14$) and matched WT mice ($n = 18$) on an odor discrimination task based on the acquisition of a Go/No-Go task (**Figure 1A**). During every session of the protocol, LFP was recorded in the OB of the WT and the ob/ob behaving mice. Neuronal signal recorded during the first 2 learning sessions (*beginner*) was compared to the signal recorded when mice reached the learning criterion (*criterion)*. 4 mice in each group were discarded from the analysis, either because they could not learn the operant task or because the LFP signal has been impaired.

In the beginning of the recordings, weight of ob/ob and WT were respectively 36.3 ± 1.1 and 21.2 ± 0.5 g. By the end of the recordings, the weight in both groups had slightly increased during behavior respectively to 39.9 ± 1.3 (paired *t*-test, $t = -2.8$, $p = 0.01$, $n = 10$ ob/ob) and 22.7 ± 0.7 (paired *t*-test, $t = -2.5$, $p = 0.02$, $n = 14$ WT).

We examined glycemia of ob/ob and WT mice after 17 h of fasting and found no significant difference in glycemia between the two groups (ob/ob, 1.22 ± 0.10 g/l, $n = 5$; WT 1.09 ± 0.20 g/l, $n = 7$; unpaired Student Test $t = 1.66$, $p = 0.13$).

Odor Discrimination Acquisition Is Modified in ob/ob Mice

We did not notice any trouble for the obese animals to move in the behavioral cage. They did not have difficulties to nose poke and to find the reward from 6 to 12 weeks of age. In a pilot experiment, we noticed that ob/ob mice were not motivated to move when the reward was water alone (data not shown). When we added sucrose (6% in water) and the motivation of ob/ob mice to perform the task became identical to WT mice in the context of the Go/No-Go task. We further explored whether motivational problems could happen in ob/ob vs. WT mice in response to peanut butter-flavored sugar pellets. We assessed motivation on a progressive ratio task that measures the amount of effort an animal is willing to exert to obtain food rewards. We did not observe any deficits in this operant behavior (cf. Supplementary Material). Consequently, all Go trials for all mice were rewarded with a sucrose solution.

Using a Go/No-Go task to assess odor discrimination performances gave the possibility to follow the behavior during intermediate stages of learning. We measured the latency between odor onset and the initiation of the nose-poke in the dipper (measured in seconds) as a behavioral marker. If the animal did not nose-poke, the latency was set at 6 s.

Results of behavioral performances in the Go/No-Go task showed variability between mice in the duration needed to reach the criterion (2 consecutive days of 90% correct choice in total, including at least 80% correct choice for the No-Go trials). This variability was identical for the two groups; animals needed between 4 and 12 sessions to reach the criterion. As illustrated on the **Figure 1D** the number of trials to criterion was not significantly different between the two groups ($p > 0.1$, Mann-Whitney's test).

We further analyzed the time course of latencies and the percentage of correct responses evolution across sessions. Each session was divided in 2 blocks of 10 trials/odor (20 trials total). To overcome the variability among individual mice, we defined 4 periods of time: *beginner* corresponded to the first 2 behavioral sessions, *criterion* corresponded to the last 2 sessions (for which the criterion has been reached). The sessions in between were split in two equal parts (half sessions are used if the total number was odd), the first half was designed as *inter1*, the second half as *inter2*. The latency has been measured for each trial of a given period, the percentage of correct response has been calculated for the entire period of time. Both have been averaged for the two odors independently.

The resulting latency curve, displayed **Figure 1B** revealed a difference in the early stage of learning between the ob/ob and the WT group. Indeed, the comparison between the latency after the Go and the No-Go stimulus showed a significant difference for *inter2* and *criterion* phases for both WT and ob/ob groups ($p < 0.0005$, Mann-Whitney's test). For the ob/ob group, the difference was already significant for *inter1* ($p < 0.01$, Mann-Whitney's test). Performances were plotted on **Figure 1C** and confirmed this effect. The percentage of correct responses for the No-Go stimulus was significantly higher for ob/ob animal since the *beginner* until *inter2* phase. In addition, no significant difference was observed for the Go stimulation, ruling out the hypothesis that ob/ob would be better at No-Go trials because they could be slower than control mice. Consequently, even if the criterion was not reached, ob/ob mice exhibited a distinct behavior for the two odors according to their value.

The Power of Odor-Induced Beta Oscillations in the OB Is Strongly Increased in ob/ob Mice

Oscillatory rhythms in the OB of awake rodents have already been described (see review in Martin and Ravel, 2014) and are characterized by a transition from high frequency gamma bursts

during exploration to beta oscillation induced by odor sampling (**Figures 2A,B**). We focused our analysis on the characteristic of the signal when the animal received the olfactory stimulation as compared to the period preceding the nose poke. The LFP recorded in the OB of ob/ob mice displayed the typical odor-induced changes: a decrease of the gamma bursts activity (60–150 Hz) and the onset of a high power oscillation at the beta frequency (15–35 Hz).

As it can be seen in time-frequency power plots averaged across all the animals for the two groups (**Figures 3Ai,ii**), the overall power variation of beta oscillations in a 15–40 Hz frequency band showed a drastic difference between WT and ob/ob mice. The power of oscillations was much stronger in ob/ob mice after the task learning. Values were then averaged for two temporal windows: the odor stimulation period (from 0 to 1 s after odor onset) and the baseline (from 2 to 1 s prior to odor onset; **Figures 3Bi,ii**). Effect of odorant, learning level and phenotype were analyzed using a Mann-Whitney test. Power during baseline and odor periods has been analyzed as repeated measurement using a paired-sample Wilcoxon signed rank test. For the two groups, odor sampling elicited beta oscillations power increase in both beginner and criterion levels (Wilcoxon test $p < 0.0001$). There was no effect of the odorant during the baseline, in any of the conditions. An effect of the odorant during the stimulation period was only observed in beginner animals, where hexanal (go stimulus) elicited a slightly higher beta power (WT $p < 0.0185$, ob/ob $p < 0.0005$). The difference was no longer significant at criterion. For both WT and ob/ob mice, odor-induced beta oscillations power was increased by learning (WT and ob/ob: $p < 0.0001$). However, even in the first two sessions (beginner), beta power during odor sampling was much higher in ob/ob mice ($p < 0.0001$) and this difference was amplified when mice had reached the criterion ($p < 0.0001$). Beta oscillations power was enhanced by 11.3 and 3.3% for the Go and No-Go stimuli respectively in beginner ob/ob animals compared with WT; in criterion animals, beta power was 47.9 and 35.8% higher in Go and No-Go stimuli respectively in ob/ob mice compared with WT.

We noticed that beta power during the baseline time period (2–1 s prior to odor onset) was also enhanced in criterion animals compared with the beginner level (WT and ob/ob: $p < 0.0001$). However, the baseline was identical between WT and ob/ob mice in both beginner and criterion animals.

The overall power increased in a time-frequency period can be explained by an increase in either the oscillatory power or the duration of beta oscillation or both. To analyze this effect thoroughly, we extracted bursts in the beta band (15–40 Hz) whose power was above a 3 STD threshold calculated from the same frequency range in the baseline period (from 2 to 1 s prior to odor onset). We then extracted the maximal power of individual significant peaks, the frequency of this maximum and the duration of individual oscillation bursts (**Figure 4A**). Indeed, we found that in criterion animals, the duration of beta bursts accounted for beta increase during odor sampling. As illustrated in **Figure 4Aii**, the duration of extracted oscillations was significantly longer in ob/ob mice compared to WT ($p < 0.005$, Mann-Whitney's test), while the difference in the maximal power of individual significant peaks did not reach significance (**Figure 4Ai**). We also observed that the frequency of beta bursts was decreased in ob/ob animals ($p < 0.005$; Mann-Whitney's test; **Figure 4Aiii**). Focusing on the duration of oscillations during odor sampling, we found that for the 2 groups of mice, a large proportion of the peaks were shorter than 400 ms. However, we noticed an increased proportion of long duration bursts in ob/ob (**Figure 4B**). Because the analysis was conducted between 0 and 1 s after odor onset, in both groups there was an overrepresentation of peaks >0.9 s. Additionally, whereas in WT burst power was not correlated with its duration, in ob/ob there was an impressive increase of power as the duration of the burst increased (**Figure 4C**). Below 400 ms duration, peaks had the same power between ob/ob and WT whereas above 500 ms, power was dramatically increased in ob/ob mice ($p < 0.0001$ Mann-Whitney's test).

To summarize, we found that LFP oscillations are stronger in the beta band (15–40 Hz) in ob/ob mice. In these animals, odorants tended to elicit longer duration burst of beta activity. In ob/ob specifically, these longer bursts reached very high energy.

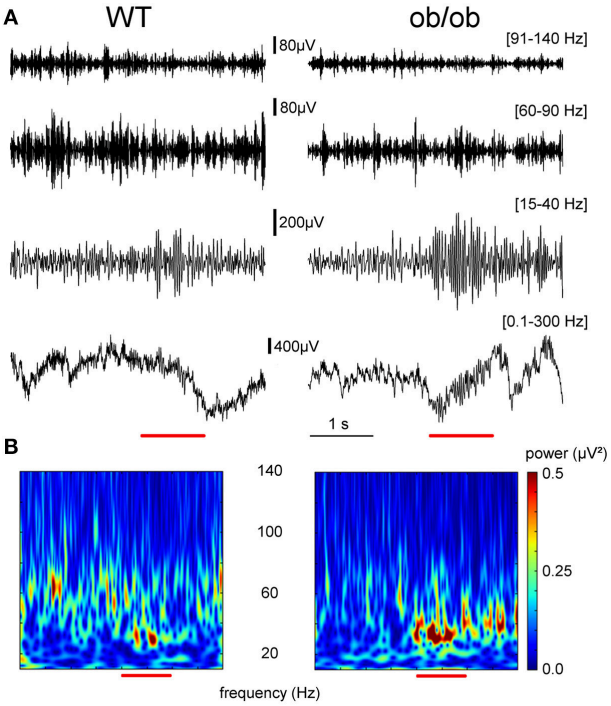

FIGURE 2 | Representative example of LFP signal recorded in WT and ob/ob mice in the OB at the criterion level. (A) Representative example of single trials LFPs recorded in the OB during odor processing (red bar) for a WT (left) and an ob/ob mouse (right). Raw LFP (0.1–300 Hz) and the corresponding filtered signals in the frequency bands of interest in this study (beta 15–40 Hz; gamma 60–90 and 91–140 Hz). The amplitude increase in the beta band was much stronger in the ob/ob mouse compared to the WT. **(B)** Time-frequency decomposition between 5 and 140 Hz showing that odor sampling transiently changed beta oscillations (15–40 Hz). Odor was butanal. *x*-Axis, time (in s); *y*-axis, frequency from 5 to 140 Hz from bottom to top. The color scale represented signal power (μV^2). The same scale was used for the 2 mice.

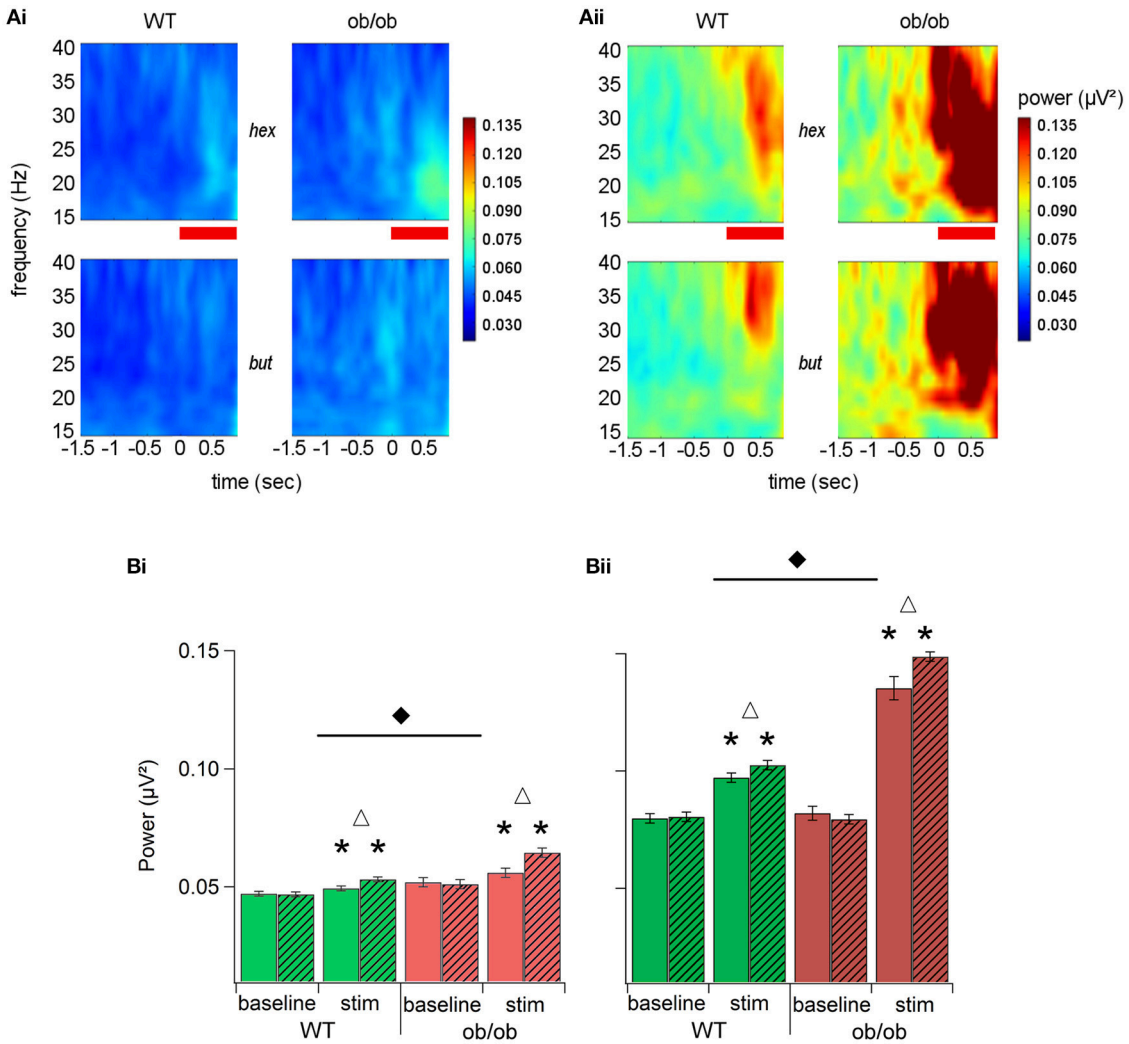

FIGURE 3 | Learning increased beta oscillatory activity in both groups but oscillations were stronger and longer in ob/ob mice. (A) Beta band (15–40 Hz) time-frequency power plots averaged across all mice (WT $n = 14$; ob/ob $n = 10$) for beginner **(Ai)** and criterion **(Aii)** levels. The two odors (hex, hexanal: Go; but, butanal: No-Go) are represented separately as marked. Zero on the time axis is the time of the nosepoke trigger of the odor. The red horizontal bar is the odor delivery period. Color scale indicates power and is consistent for the two panels. **(B)** Mean power (±SEM) of the beta band (15–40 Hz) averaged across all mice (WT $n = 14$; ob/ob $n = 10$) for beginner **(Bi)** and criterion **(Bii)** levels for the two odorants of the pair (empty bars: hexanal; hatched bars: butanal). All mean values during odor stimulation were significantly above the mean for the baseline period (*Wilcoxon test $p < 0.0001$). Delta indicates significant differences between the 2 odors. Black diamond denotes a significant difference between WT and ob/ob mice.

Spontaneous Gamma Bursts Are Shifted toward Higher Frequencies in ob/ob Mice

When the animal was freely exploring the arena in the absence of odorant stimulation, LFP oscillatory activity was characterized by bursts of gamma activity superimposed on slow deflections of the signal corresponding to the respiration-related rhythm (Nguyen Chi et al., 2016). We observed that this relation to respiration was preserved in ob/ob mice.

Because of the transient nature of gamma oscillations in the OB, we extracted all the bursts whose power was above a threshold set at 2 STD of the mean power value in the time-frequency region of interest (baseline, 60–150 Hz). We then extracted the maximal power of individual significant peaks and the frequency of the maximum.

In both WT and ob/ob, the median number of significant peak was 3 s^{-1}. However, the analysis of the frequency and power of individual gamma bursts during baseline period revealed an alteration in ob/ob mice. The repartition of peaks frequency was changed (Test X^2; $p < 0.0001$). As illustrated in **Figure 5A** in WT mice, most of the peaks had a frequency under 90 Hz. In ob/ob mice, the repartition of peaks turned bimodal with a large proportion of gamma peaks also between 90 and 115 Hz. This shift of activity was confirmed when plotting the power of peaks as a function of their frequency. The power of peaks ranging from

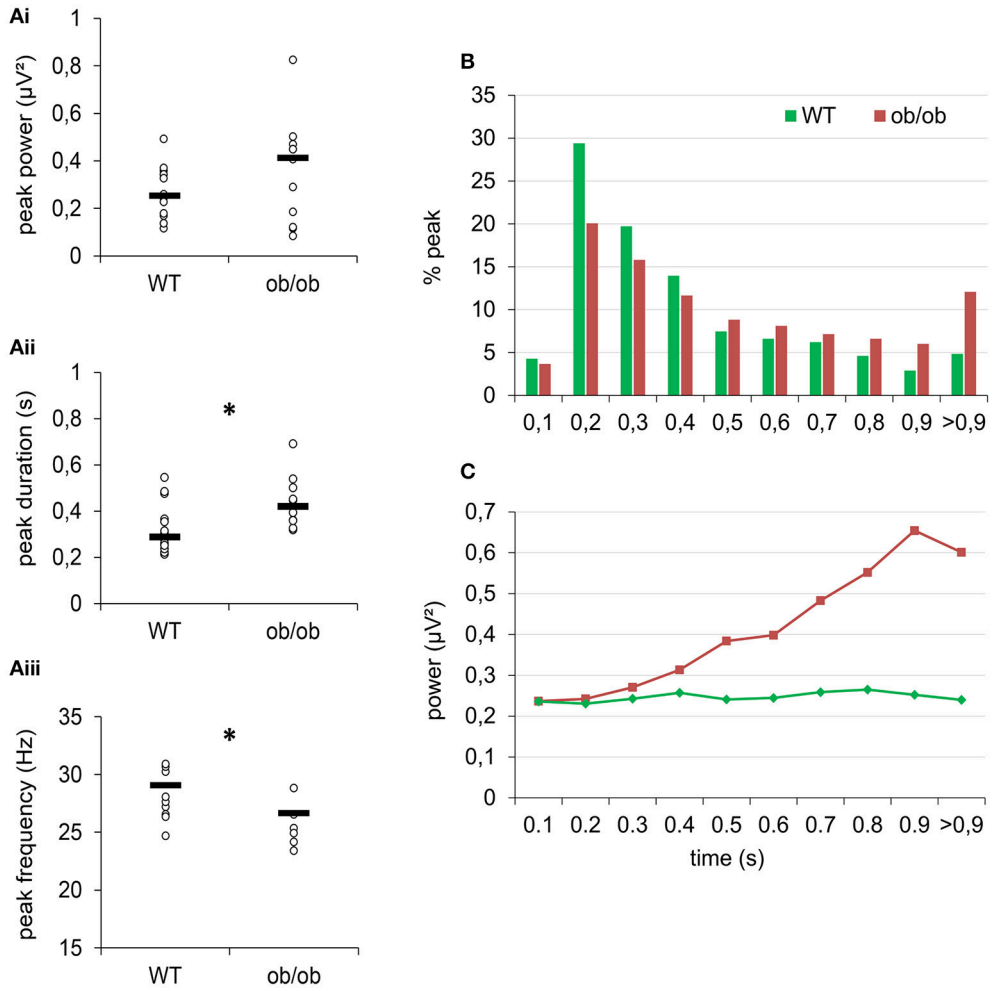

FIGURE 4 | After learning, odor sampling elicited longer and stronger oscillatory peaks in the beta band in ob/ob mice. (A) Comparison of peaks power **(Ai)**, duration **(Aii)**, and frequency **(Aiii)** of odor-evoked beta bursts at criterion between WT and ob/ob mice. Each symbol denotes one mouse; hyphen is the median. *$p < 0.005$ Mann-Whitney. **(B)** Distribution of the peaks of beta oscillations according to their duration for the 2 groups. For both groups, most of beta peaks lasted for 200–400 ms. However, there was an overrepresentation of long peaks (>900 ms) in ob/ob animals. **(C)** Mean power of peaks plotted as a function of beta peaks duration shows a dramatic increase of power for peaks longer than 0.5 s in ob/ob mice but not in WT. *$p < 0.0001$ Mann-Whitney test.

90 to 115 Hz was higher in ob/ob mice compared to WT mice. The difference was significant for [91–95 Hz], [101–105 Hz], [106–110 Hz], and [131–135 Hz] ($p < 0.01$, Mann-Whitney's test, **Figure 5B**). This difference, illustrated for the beginner session was similar after training (criterion sessions). However, results showed that gamma power during ongoing activity was enhanced by learning (**Figure 5C**). We further studied this effect by splitting the gamma band into two frequency bands according to the effect described above. Then we examined power of peaks ranging from [60–90 Hz] and from [91–150 Hz]. As expected from the repartition, for the 2 frequency bands and the two learning levels, gamma power was significantly different between WT and ob/ob animals (Mann-Whitney $p < 0.0001$ except for criterion level between [91–150 Hz], $p = 0.0294$). In the two frequency bands, gamma power increased after learning for both phenotypes (Mann-Whitney $p < 0.0001$).

DISCUSSION

Here we tested the neuronal network underlying olfactory processing using LFP recording in awake mice engaged in learning. We showed that in behaving unrestrained mice performing a Go/No-Go task for odor discrimination (i) ob/ob mice were more efficient to discriminate the two odors according to their reward in the early stages of the task (ii) OB oscillations were under the dependence of leptin availability because in ob/ob mice both beta and gamma bands, two markers of the olfactory network dynamics were modified in power and frequency.

Very few data are available on the link between leptin and olfactory performances in rodents. Intracerebroventricular injection of leptin has been shown to decrease the olfactory sensitivity in rats in the context of a conditioned olfactory aversion (Julliard et al., 2007). Consistently, when challenged in

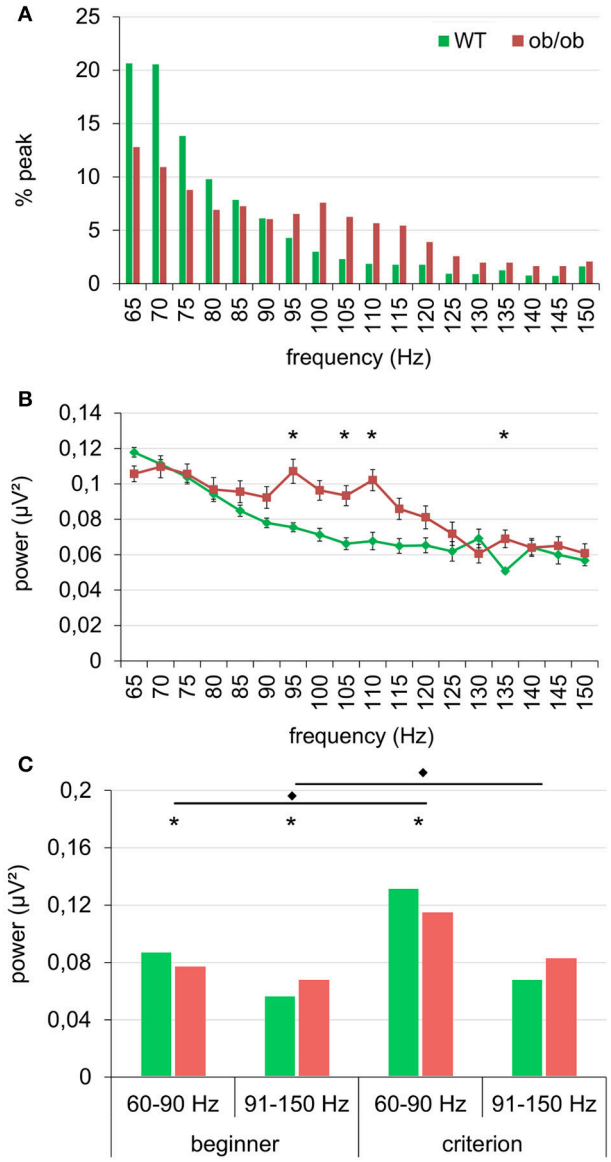

FIGURE 5 | Gamma peaks were shifted in frequency in ob/ob mice. (A) The distribution of the peaks in the gamma frequency range became bimodal and was shifted toward higher frequencies (90–120 Hz) in ob/ob mice compared to WT mice. **(B)** This shift was also illustrated by mean power of peaks plotted as a function of their frequency (*$p < 0.01$ Mann-Whitney test). **(A,B)** are for beginner condition, similar effect was observed at the criterion. **(C)** Power of the peaks (median) in the gamma range for two frequency bands [60–90 Hz] and [91–150 Hz] confirmed the gamma shift toward higher values in ob/ob animals and denotes a general increase in gamma power after learning (*$p < 0.01$ Mann-Whitney test between WT and ob/ob; *$p < 0.0001$ Mann-Whitney test between beginner and criterion).

a task where they have to find a buried palatable food, ob/ob mice lacking leptin are faster in spontaneously retrieving the odorized food (Getchell et al., 2006). Our results in the odor discrimination task are in agreement with this gain of function, importantly extending it to learning: at least in the beginning of learning and because ob/ob mice have a faster transition between the beginner and the intermediate learning stage, they are quicker to discriminate between the two odors. However, in fine, ob/ob and WT mice spent the same amount of trials to reach the set criterion. Discrimination time reaction in a Go/No-Go task is determined not only by sensory processing but is also influenced by other factors such as motor behavior and motivational effects (Friedrich, 2006; Frederick et al., 2011). Despite the fact that we found no difference between ob/ob and WT mice in the context of a progressive ratio task where mice are required to press a lever to obtain a palatable reward, we cannot definitely rule out that leptin deficiency may have increased the rewarding post-ingesting effect of sucrose in the ob/ob mice (Domingos et al., 2011).

To further explore the role of leptin in OB neuronal oscillations and plasticity with learning we recorded LFP in the OB network of ob/ob compared to WT mice. In the olfactory system of awake rodents, odor sampling leads to a decrease in the gamma (60–100 Hz) bursting activity which is replaced by an increased in power of oscillations in the beta band (15–40 Hz) (Martin and Ravel, 2014). This shift in the oscillatory dynamics between gamma and beta frequencies is enhanced when odors become meaningful by associative learning and has been reported in numerous studies in the OB and piriform cortex (Martin et al., 2004; Chapuis et al., 2009; Lepousez and Lledo, 2013). In ob/ob mice, for both rewarded and non-rewarded odors, we found a very strong elevation of beta oscillation during odor sampling in addition to a shift in the frequency of gamma bursts. These specific changes in the OB temporal dynamics suggested profound modifications in the network involved in odor processing. Indeed, we have previously shown that beta power is highly dependent on CF onto the OB, most of them coming from the olfactory cortex. Pharmacological blockade of this CF abolished beta oscillation elicited by odor sampling (Martin et al., 2006). Therefore, according to previous studies (Pager et al., 1972; Chabaud et al., 2000), we hypothesize that the increase in the beta activity in ob/ob mice can be due to a modification in the CF-dependent regulation to the OB. Leptin would modulate OB physiology by acting directly or indirectly on CF.

CB1 receptors were shown to be major determinants of the OB electrical activity in fasted mice. Indeed, CB1 receptor activation selectively reduces the cortical inhibition of mitral cell spiking activity (Soria-Gómez et al., 2014). It has been suggested that leptin could act in interaction with CB1 receptors in the brain. In the hypothalamus, leptin appears to decrease food intake partly by reducing the levels of endogenous cannabinoids (Di Marzo et al., 2001). As reported for leptin receptor in ob/ob mice (Huang et al., 1997), a higher level of CB1R is found in obese Zucker rats characterized by a mutation in the leptin receptor suggesting that leptin interferes with CB1 receptor upregulation (Thanos et al., 2008). Thus, in the OB, leptin receptor activity may tune the amplitude of the oscillations and odorant processing via CB1 receptors. CB1 receptor upregulation would decrease control of CF inputs onto granule cells. This could result in changes in inhibitory tone on MC and therefore enhance oscillatory patterns. The fact that odorant-induced beta oscillations episodes were longer and stronger in ob/ob

mice is in favor of an alteration in inhibitory processes. Why animals with leptin pathway dysfunction have a propensity to develop greater sensitivity to odors? Leptin having anorectic properties, it can be hypothesized that this hormone may have an inhibitory effect on the olfactory pathways necessary to search for food. The lack of the hormone—as is the case in ob/ob mice—will exacerbate challenging pathways in search of food as olfaction.

In the hypothalamus, a specific CB1-leptin interaction involving astrocytes was shown to regulate neuronal circuits and feeding (Bosier et al., 2013; Kim et al., 2014). Indeed, in the OB the presence of obR was shown on glomerular and granular astrocytes (Prud'homme et al., 2009). In addition, we have previously observed that a modification in astrocytic activity impaired oscillatory activity in the mouse OB (Martin et al., 2012). In fact, in GLAST-deficient mice, we also described a shift in gamma bursts high frequency (Martin et al., 2012). Within the OB, gamma oscillations are supported by the reciprocal synapse between mitral and granule cells (Lagier et al., 2007; David et al., 2009; Lepousez and Lledo, 2013). Taking all these facts together, specific interactions between leptin and CB1 involving astrocytes could be a key to explain changes in OB neuronal oscillations in ob/ob mice and will be the subject of future work.

In conclusion, our study demonstrated for the first time profound changes in oscillatory activities in ob/ob mice. It revealed that beside its known action as energy-related anorectic signal, leptin might directly modulate neural encoding of potent food-related cues such as odors hence potentially encoding food rewarding value (Trellakis et al., 2011). These results in ob/ob mice represent the first step in the functional study of leptin-dependent pathways within the OB and pave the way for the study of leptin as a major regulator of the OB for food odor processing in the context of feeding.

AUTHOR CONTRIBUTIONS

Design of experiments: YC, NM, HG, and CM; data collection: YC, AE; data analysis YC, AE, HG, and CM, data interpretation: YC, SL, NM, HG, and CM writing of the paper ChM, SL, NM, HG, and CM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00002/full#supplementary-material

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REVIEW
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Nutrient Sensing: Another Chemosensitivity of the Olfactory System

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Olfaction is a major sensory modality involved in real time perception of the chemical composition of the external environment. Olfaction favors anticipation and rapid adaptation of behavioral responses necessary for animal survival. Furthermore, recent studies have demonstrated that there is a direct action of metabolic peptides on the olfactory network. Orexigenic peptides such as ghrelin and orexin increase olfactory sensitivity, which in turn, is decreased by anorexigenic hormones such as insulin and leptin. In addition to peptides, nutrients can play a key role on neuronal activity. Very little is known about nutrient sensing in olfactory areas. Nutrients, such as carbohydrates, amino acids, and lipids, could play a key role in modulating olfactory sensitivity to adjust feeding behavior according to metabolic need. Here we summarize recent findings on nutrient-sensing neurons in olfactory areas and delineate the limits of our knowledge on this topic. The present review opens new lines of investigations on the relationship between olfaction and food intake, which could contribute to determining the etiology of metabolic disorders.

Keywords: nutrient sensing, olfaction, piriform cortex, transporter, receptor, food intake, obesity, type 2 diabetes

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THE OLFACTORY SYSTEM IS AN INTERFACE

According to its anatomical location, the olfactory system is well poised to be an interface, with the ability to gather and process information simultaneously from the external and internal environment.

Interaction with the External Environment

The traditional function of the olfactory system is to sense the external chemical world. Odors are inhaled directly into the nose following an orthonasal pathway, or come from the back part of the mouth following a retronasal pathway. Both pathways lead odors to the posterior part of the nasal cavity. Odors bind to protein receptors located in the ciliary membrane of olfactory sensory neurons (OSNs) within the olfactory epithelium (OE). Each OSN expresses only one type of olfactory receptor (Malnic et al., 1999; Serizawa et al., 2003). Odor/receptor association selectively activates OSNs in the OE. All OSNs expressing the same odorant receptor project their axons to one or two olfactory bulb (OB) glomeruli where OSN axons synapse with the dendrites of mitral cells (MCs); the second order olfactory neurons

(Ressler et al., 1994; Vassar et al., 1994; Breer et al., 2006). The electrical signal is then transmitted to neuronal networks in the piriform cortex (PC). Olfaction thereby informs the central nervous system in real time about the chemical composition of the external environment prior to any visual or tactile information. This event allows the animal to anticipate and rapidly adapt its behavior when seeking food or when engaging in social or sexual behavior.

Interaction with the Internal Environment

The hypothalamus is the main central actor in food intake regulation. Internal signals carried by the blood inform various central areas about the body's fuel availability, which in turn implement appropriate behavioral and metabolic responses to physiological requirements. Orexigenic and anorexigenic signals, respectively, stimulate or inhibit food intake by modulating neuronal activity of hypothalamic nuclei. During fasting, the hypothalamus induces food intake in response to nutrient scarcity and high level of ghrelin released by the stomach. Alternatively, the hypothalamus suppresses feeding behavior when it detects insulin secretion from the pancreas, leptin secretion from the adipose tissue, and nutrient abundance (Blouet and Schwartz, 2010; Berthoud, 2011). Interestingly, the olfactory system is also becoming widely considered as an active sensor of internal signaling (hormones, micronutrients availability). Olfactory structures like the OE, OB, and PC (Palouzier-Paulignan et al., 2012) express high levels of various hormone receptors (insulin, leptin, ghrelin, CCK) similar to that of the hypothalamus (**Figure 1**). When targeting their receptors, metabolic hormones modulate the electrical activity of olfactory networks (Fadool et al., 2000, 2011; Apelbaum et al., 2005; Hardy et al., 2005; Lacroix et al., 2008; Savigner et al., 2009; Kuczewski et al., 2014). OB neurons respond not only to peptides, but they also respond to glucose and express molecular hallmarks of glucose sensing cells (Tucker et al., 2010, 2013; Aimé et al., 2014; Al Koborssy et al., 2014; Kovach et al., 2016).

The metabolic sensing function of the OB is consistent with its high density of capillary network (Chaigneau et al., 2007) and its vascular properties. The blood brain barrier of the OB is not as tight as it is in the cerebral cortex or other brain regions (Ueno et al., 1991, 1996), indicating that blood-borne metabolic signals can enter the OB more easily than other brain regions. The permeable blood barrier facilitates transport of intravascular macromolecules, including nutrients and peripheral hormones, and their direct action on the OB. This enhanced permeability allows adaptation of olfactory perception to the physiological state: highly sensitive when the animal is fasted and needs to find food, and slightly sensitive when the animal is satiated (Aimé et al., 2007, 2012; Julliard et al., 2007; Prud'homme et al., 2009; Tong et al., 2011). Based upon its sensitivity to metabolic hormones and glucose availability, the olfactory system is proposed to be a metabolic sensor.

The present review provides an updated outlook of nutrient sensing in olfactory structures. We argue that in addition to being glucose-sensitive (Tucker et al., 2010, 2013; Aimé et al., 2014; Al Koborssy et al., 2014; Kovach et al., 2016) olfactory structures are sensors of amino acids (AAs) and potentially of fatty acid (FA) content of the internal medium.

TRANSMEMBRANE PROTEIN FAMILIES INVOLVED IN NUTRIENT SENSING

In contrast to unicellular organisms, most eukaryotic cells are not directly exposed to changes in environmental nutrients. Nevertheless, nutrient homeostasis is essential for all living organisms to maintain constant fuel supply despite discontinuity in food intake. Nutrient scarcity and abundance exert a strong pressure on the selection of efficient mechanisms for nutrient sensing in mammalian cells including central neurons. However, the molecular nature of brain nutrient sensors has only recently started to be deciphered. The present review focuses on sensors that are present in olfactory areas. In particular, we present two major sensing mechanisms that involve either the family of solute carrier (SLC) transporters (called T in **Figure 2**) or receptors having seven or two transmembrane domains (called R in **Figure 2**).

In the first mechanism, the sensed molecule is transported intracellularly. Numerous transmembrane protein transporters belonging to the SLC superfamily have been associated with nutrient sensing that control feeding, energy expenditure, and counterregulation (Marty et al., 2007; Gonzalez et al., 2009; Routh, 2010; Broer, 2014). The SLC superfamily mediates passage of nutrients across the phospholipid bilayer via passive transport, in which the nutrient moves down its concentration gradient, or via active transport (or co-transport) that couples the movement of the nutrient to that of another molecule or ion crossing the membrane either in the same (symporter) or opposite direction (antiporter or exchanger). As a result, the membrane potential can be modulated directly when the sensed molecule is co-transported with ions (electrogenic transport) or indirectly when the sensed molecule activates an intracellular cascade which, in turn, modulates ion channel permeability (non-electrogenic transport).

In the second sensing mechanism, the sensed molecule binds to its transmembrane receptor and activates an intracellular cascade to depolarize the membrane through activation of Na^+ and/or Ca^{2+} inflow or inhibition of K^+ conductance (Lindemann, 2001; Chaudhari and Roper, 2010). In nutrient sensing, the most important transmembrane receptors belong to

Abbreviations: CoA, Acyl-coenzyme A; AA, Amino acid; AON, Anterior olfactory nucleus; APC, Anterior piriform cortex; CD36, Cluster of Differentiation 36; DAG, Diacylglycerol; iEPL, Internal External plexiform layer; FA, Fatty acid; FAT, Fatty acid translocase; FATP, Fatty acid transport proteins; GCN2, General amino acid control non-derepressible 2; GE, Glucose-excited; GI, Glucose-inhibited; GLUT, Glucose transporter; GPCR or GPR, G-protein-coupled receptor; iEPL, Inner part of the external plexiform layer; IP_3, Inositol 1,4,5-trisphosphate; IR, Insulin receptor; KCC2, K+/Cl- co-transporter; mTORC1, Mammalian target of rapamycin complex 1; MCs, Mitral cells; OB, Olfactory bulb; OE, Olfactory epithelium; OSN, Olfactory sensory neuron; OT, Olfactory tubercle; PIP_2, Phosphatidylinositol 4,5-bisphosphate; PLC, Phospholipase C; PC, Piriform cortex; PUFA, Polyunsaturated fatty acid; 7TM, Seven transmembrane domains; SGLT, Sodium-dependent glucose transporter; SLC, Solute carrier; TRPC, Transient receptor potential cation channel subfamily C; TRPM, Transient receptor potential cation channel subfamily M.

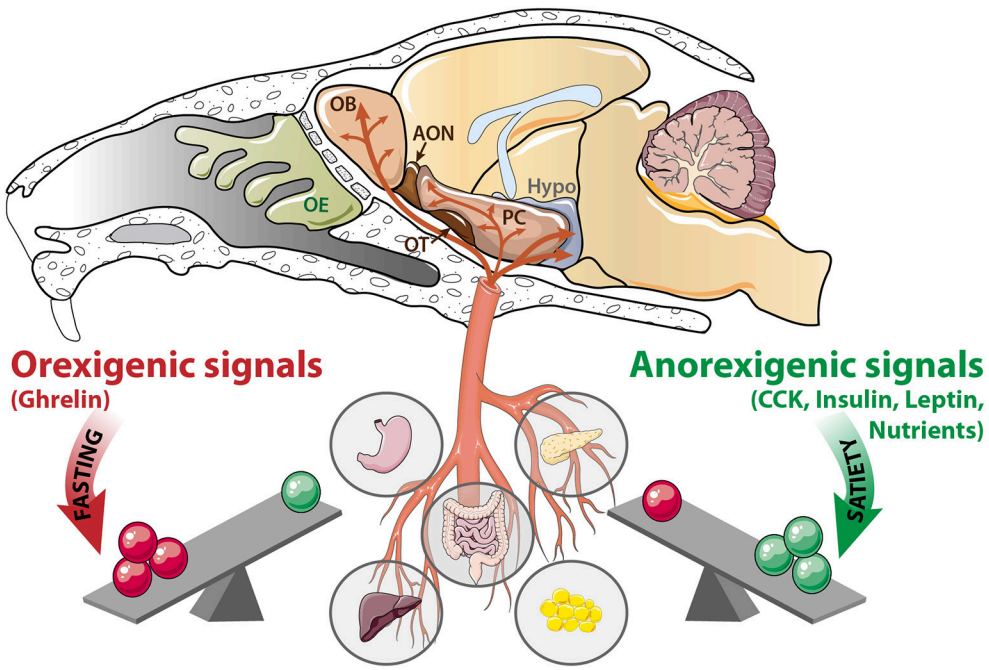

FIGURE 1 | The olfactory system is a metabolic sensor like the hypothalamus. According to the nutritional status, a balance exists between peripheral signals delivered by the stomach, intestine, liver, pancreas, and adipose tissue. During fasting, orexigenic signals (ghrelin, and nutrients scarcity) prevail. In contrast, during satiation, anorexigenic signals (CCK, insulin, leptin and nutrients abundance) are predominant. These signaling molecules reach the central nervous system via the blood flow, where they target the hypothalamus (Hypo) as well as a variety of olfactory structures: OE, olfactory epithelium; OB, olfactory bulb; AON, anterior olfactory nucleus, OT, olfactory tubercle; PC: piriform cortex; CCK, cholecystokinin.

the seven transmembrane (7TM) G protein–coupled receptors (GPCRs) family and are activated by glucose, AAs, or FAs. These 7TM receptors are expressed in central nervous areas involved in energy homeostasis regulation (Wellendorph et al., 2010). The 7TM receptors exist across the phospholipid bilayer as homodimers, heterodimers, or monomers. It is noteworthy that a 2TM receptor called cluster of differentiation 36 (CD36), is often associated with FAs transporters in the hypothalamus (Doege and Stahl, 2006; Magnan et al., 2015).

GLUCOSE SENSING

Physiological Role of Glucose Supply to the Brain

Glucose is the primary metabolic substrate for the brain and a continuous supply of glucose is required for normal neuronal function (Mergenthaler et al., 2013). The brain accounts for 2% of the total body mass but requires 10 times more energy in the resting state compared to other energy consumption needs of the body (Mink et al., 1981; Molina and DiMaio, 2012). Glucose metabolism provides the fuel for physiological brain function through the generation of ATP that serves for the basic maintenance of cellular processes such as cytoskeletal dynamics, DNA repair, protein turnover, and growth. More specifically, during neuronal activation, the brain consumes a lot of energy in order to maintain the turnover of glutamate through metabolic neuron-astrocyte interactions (Magistretti and Allaman, 2015). Furthermore, 80% of total energy consumption fuels the Na^+/K^+ ATPase pump but <10% is used to recycle second messengers and neurotransmitters (Laughlin, 2001).

Glucose supply is critical for physiology, therefore a tight regulation between supply and demand is required. Several brain areas, such as the hypothalamus, brainstem, amygdala, septum, hippocampus, cortex, and OB contain glucose sensing neurons (Anand et al., 1964; Oomura et al., 1969; Ritter et al., 1981; Nakano et al., 1986; Shoji, 1992; Balfour et al., 2006; Tucker et al., 2013). These specialized neurons respond to local fluctuations in extracellular glucose levels, and modulate their mean firing rate accordingly. Glucose sensing neurons have been classified as "glucose-excited" (GE) or "glucose-inhibited" (GI) depending on whether they increase or decrease action potential frequency in response to extracellular glucose variations (McCrimmon, 2008; Gonzalez et al., 2009). GE and GI neurons integrate fluctuations in whole-body metabolic signals related to feeding behavior (Routh et al., 2007).

Several transporters, receptors, and ion channels are expressed in glucose sensing neurons of olfactory structures. Our laboratories and others have studied the role of the sodium-dependent glucose transporters (SGLTs), glucose transporters (GLUTs), potassium channels, and the insulin receptor (IR) in sensing glucose.

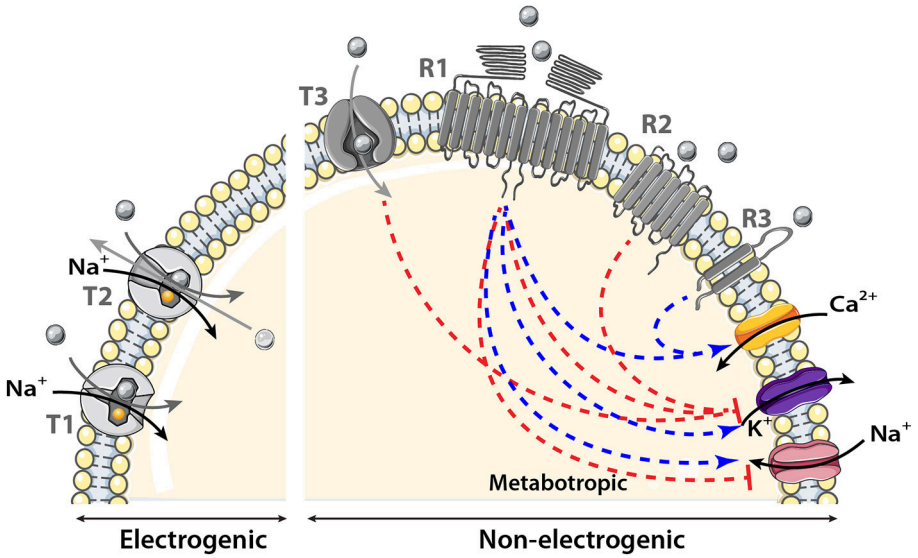

FIGURE 2 | Schematic model showing the transmembrane proteins involved in nutrient sensing. The main transporter (T) family involved in nutrient sensing is the solute carrier (SLC) transporter family. It couples the movement of the nutrient (gray circle) to that of another molecule or ion crossing the membrane either in the same (symporter) named T1 in the figure or opposite direction (antiporter or exchanger) named T2 in the model. Nutrient influx down SLC transporters is called electrogenic when associated with a net inward of ion of Na^+ of sufficient magnitude to cause direct membrane depolarization. Transport is non-electrogenic when it activates intracellular cascades that in turn depolarizes the membrane for example via K^+ conductance inhibition. The two receptor (R) families involved in nutrient sensing are: the large receptor family of seven transmembrane domains (**7TM)** named R1 and R2 in the figure and the smaller family of two transmembrane domains (**2TM**) named R3 in the schematic model. The main receptor family is composed of 7TM it could be observed as heterodimer, homodimer (R1) or monomer (R2). Nutrients binding to their receptors activate an intracellular cascade which induces membrane depolarization by activating (blue arrow) a Na^+ influx or by inhibiting (red line) K^+ conductance or hyperpolarization by the reverse events. Metabotropic (via intracellular cascades) activation and inhibition of ion channels induced by nutrients are represented by the blue and red dotted lines respectively.

Sensing Role of Glucose in Olfactory Structures: Molecular Hallmarks

Glucose Transporters Expressed in Olfactory Structures

Electrogenic solute carrier transporter (SGLT1)

The family of sodium-dependent glucose transporters (SGLTs), also named SLC5, belongs to the SLC super family and uses a Na^+ gradient to transport glucose against its concentration gradient into the cell. To date, six SGLTs isoforms have been identified (Wright and Turk, 2004). SGLT1 can modify its conformation to first release the two Na^+ ions intracellularly while transporting glucose against its concentration gradient albeit in a symport orientation (**Figure 3**).

In the brain, SGLT1 has been found mainly in the hypothalamus, hippocampus, amygdala and OB (Kang et al., 2004; Yu et al., 2010; Aimé et al., 2014; Al Koborssy et al., 2014). In the OB, we found strong staining of SGLT1 in the inner part of the external plexiform layer (iEPL), in some mitral cells (MCs) and in some glomeruli (Al Koborssy et al., 2014). The iEPL is the site of reciprocal dendro-dendritic synapses between the secondary dendrites of MCs and the dendritic spines of inhibitory granule cells; this inhibitory interaction modulates odor information including olfactory discrimination (Yokoi et al., 1995; Lledo et al., 2005; Abraham et al., 2010). The availability of inhibitory control over MCs combined with the presence of rapidly activating SGLTs in the iEPL could explain the inhibitory response to glucose observed in the GI class of MCs (Tucker et al., 2013).

Non-electrogenic solute carrier transporter (GLUT4)

The Na^+-independent GLUTs family (gene family *slc*2a) transports glucose across biological membranes. GLUTs comprise 14 family members and exhibit diverse substrate and tissue specificity resulting in distinct functional characteristics. GLUT1 exists as two isoforms in the brain and is exclusively expressed in endothelial cells and astrocytes. GLUT3 is localized to the neuropil and is largely absent from neuronal cell bodies (McCall et al., 1994; Gerhart et al., 1995) while GLUT4 exhibits a somato-dendritic labeling. The more discrete presence of GLUT4 compared with other GLUTs suggests that GLUT4 may be involved in highly specialized activities in the central nervous system. GLUT4 is consistently colocalized with IR and glucose transport through GLUT4 is the rate-limiting step in insulin-stimulated glucose uptake in the brain including olfactory areas (Alquier et al., 2006). Interestingly, 75% of GE neurons in the central nervous system coexpress GLUT4 and the IR mRNA (Kang et al., 2004).

The olfactory system has been found to express GLUT1 in the OE (Nunez-Parra et al., 2011), whereas GLUT1, GLUT3, and GLUT4 have been reported in the central olfactory areas (Brant et al., 1993; Leloup et al., 1996; El Messari et al., 1998, 2002; Vannucci et al., 1998; Dobrogowska and Vorbrodt, 1999;

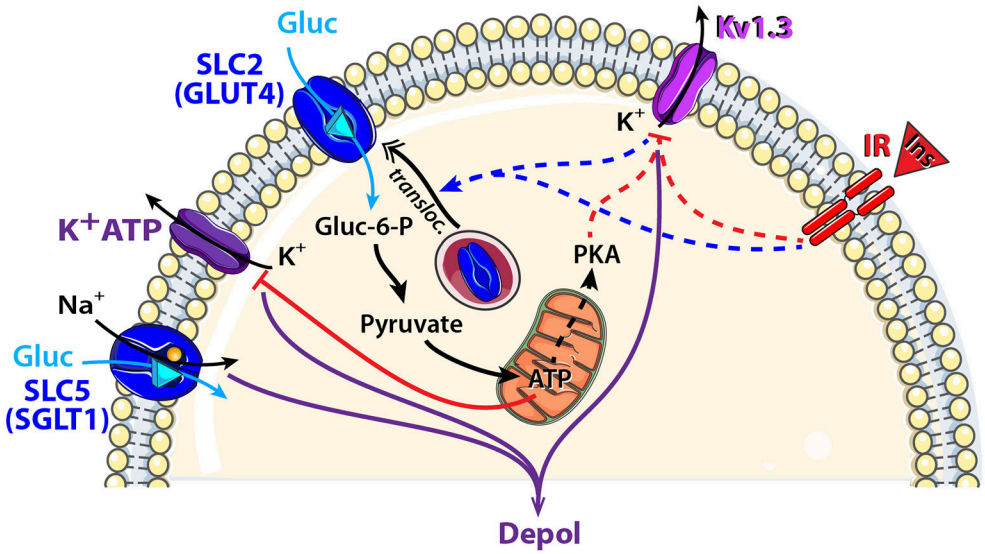

FIGURE 3 | Schematic model showing glucose sensing signaling pathways that might modulate neuronal activity of central olfactory areas. Two types of glucose transporters and their associated downstream cellular processes are observed in central olfactory areas. SGLT1, located in the OB, is electrogenic and combines glucose (Gluc: blue triangle) translocation with an influx of Na^+. GLUT4, located mainly in the OB and PC, is non-electrogenic and is associated with the insulin pathway. Indeed, insulin (Ins, red triangle) binding to its receptor (IR: insulin receptor) depolarizes MCs through Kv1.3 channel closure and induces GLUT4 translocation to the membrane. Glucose intake increases as well as the mitochondrial production of ATP and the cytosolic protein kinase A (PKA). Activation: blue arrow, inhibition: red line. Direct and indirect action of one molecule: full and dotted line respectively.

Choeiri et al., 2002; Al Koborssy et al., 2014). GLUT4 and IR are found to be localized in the main central olfactory areas such as the OB, PC, anterior olfactory nucleus (AON), and olfactory tubercle (OT) (Unger et al., 1989; Marks et al., 1990; El Messari et al., 1998; Schulingkamp et al., 2000; Alquier et al., 2006; Aimé et al., 2012, 2014). In a previous study, we have shown that GLUT4 is co-localized with IR in MCs and glomeruli of the OB. Interestingly, subcellular localization of GLUT4 is modulated by the feeding state. During the postprandial period when glucose levels in the blood are high, GLUT4 is found on the plasma membrane of dendritic processes. Following a fast however, it becomes internalized into the cytoplasm (Al Koborssy et al., 2014).

The dynamic expression of GLUT4 within MCs can be regulated by two complementary mechanisms (**Figure 3**). First, we observed that the feeding state-dependent modulation of GLUT4 subcellular localization in the OB correlates with the feeding state-dependent fluctuations of insulin levels in the OB as insulin was 2 fold higher in fed rats compared to fasted rats (Aimé et al., 2012). We infer that insulin levels increase in the OB during satiety to stimulate translocation of GLUT4 storage vesicles to the plasma membrane thereby increasing glucose uptake. Second, subcellular expression of GLUT4 can be regulated by the voltage-dependent potassium channel, Kv1.3 (Xu et al., 2004; Kovach et al., 2016). Blocking Kv1.3 conductance by applying a specific inhibitor (margatoxin) to cultured adipocytes or by co-transfecting GLUT4 and a non-conducting pore form of the channel in human embryonic kidney cells, increases plasma membrane expression of GLUT4 (Xu et al., 2004; Kovach et al., 2016). Gene-targeted deletion of Kv1.3 channel renders glucose-sensitive MCs non-responsive to glucose modulation in terms of action potential firing frequency (Tucker et al., 2013). Kv1.3 was further hypothesized to act as an insulin receptor substrate in MCs whereby IR activation phosphorylates the channel and suppresses its peak current (Fadool et al., 2000). It results that insulin-dependent or -independent blockade of Kv1.3 increases glucose translocation to the membrane.

While GLUT4 is highly expressed in MCs, and these neurons are clearly able to sense changes in glucose concentration either experimentally or evoked by nutritional state *in vivo*, the steps linking glucose entry to the change in firing pattern of MCs are yet unknown. We speculate that glucose sensing of MCs might use similar molecular means as reported for glucose sensing of the hypothalamus (Ashford et al., 1990; Spanswick et al., 1997; Ashcroft and Gribble, 1999; Song et al., 2001). In addition to K_{ATP}, other transporters like the Na^+/K^+ ATPase pump (Oomura, 1983; Silver and Erecinska, 1998), and the cystic fibrosis transmembrane conductance regulator chloride channel (Hwang and Sheppard, 1999; Song et al., 2001) could elicit either depolarization or hyperpolarization of a neuron during extracellular glucose fluctuation.

Further studies are required to elucidate (i) if glucose transport across MCs recruits an electrogenic symport of Na^+, (ii) if the metabolic product of glucose (ATP) acts on downstream ion channels similar to mechanisms observed in the hypothalamus or (iii) if byproducts of glucose metabolism could phosphorylate Kv1.3 through ATP, cAMP, or PKA (Lewis and Cahalan, 1995; Dalle et al., 2013).

Metabolic Dysfunction and Glucose Sensors in Olfactory Areas

A variety of functions have been suggested for central glucose sensing neurons. Glucose sensing neurons are involved (i) in maintaining local energy requirements for synaptic transmission and (ii) in regulating whole body energy and glucose homeostasis. Glucose not only serves as a metabolic substrate but also alters neuronal activity linked to metabolism. Therefore, it's suggested that correct functioning of glucose sensing neurons would be essential to prevent metabolic disorders such as obesity and Type 2 diabetes mellitus but also stroke and other neurodegenerative disorders where neuronal energy supply is disrupted (Routh et al., 2007).

Central olfactory areas such as the OB and PC, have an expensive energy budget in terms of glucose metabolism, which is high during odor stimulation and increases further during coding and processing of olfactory information (Nawroth et al., 2007; Gire et al., 2013; Litaudon et al., 2017). Given that, we previously established a link between feeding states and olfactory performance, and adding the dynamic changes in GLUT4 expression, insulin levels, and the numerous metabolic hormones present in the OB, we suggest that glucose sensing neurons are keys regulators of metabolic-dependent olfactory behavior.

In rodents, the concentration, expression, and activity of several molecules involved in glucose-sensing in olfactory areas are not only modified with feeding behavior but they are also altered by metabolic pathologies and their subsequent nutritional imbalance. In the OB, insulin concentration and GLUT4 expression are feeding-dependent but SGLT1 and IR expression are not (Aimé et al., 2012; Al Koborssy et al., 2014). In commonly used rodent models of obesity and type 2 diabetes, insulin concentration is elevated and SGLT1 is upregulated in the OB. Moreover, IR expression is down regulated but GLUT4 remained affected in both the OB and PC (Livingston et al., 1993; Vannucci et al., 1998; Aimé et al., 2014). Rodent models of obesity further display increased olfactory sensitivity and discrimination (Aimé et al., 2014; Chelminski et al., 2017).

We propose that dysregulation of glucose sensing markers could induce an increase in olfactory sensitivity which could lead to hyperphagia and metabolic disorders. These results suggest that dysfunctional glucose sensing neurons in the OB could alter olfactory pathways crucial to the regulation of food intake.

AMINO ACID SENSING

Physiological Role of Amino Acid Supply to the Brain

Amino acids (AAs) play a key physiological role as building blocks of proteins. Proteins not only play a structural role in the organism but they are involved in various metabolic processes, including enzymatic reactions. Among the 20 AAs that serve for protein synthesis, 10 are referred to as the essential AAs because they are acquired only from the diet and cannot be stored in the body. AA supply requires numerous membrane transporters and receptors that are tissue specific. Each carrier recognizes several AAs having structural similarities. In this manner, one AA is transported inside cells through multiple carriers with overlapping specificities (Taylor, 2014).

AAs are key regulators of metabolism (Wu, 2009). Homeostatic regulation of AA level is necessary to adapt AA concentration (essential and non-essential AAs) to physiological body requirements. In order to maintain an adequate AA supply, the hypothalamus senses AA notably through leucine detection that signals AA abundance and directly regulates food intake. Leucine intake activates the mammalian target of rapamycin complex 1 (mTORC1) and inhibits AMP-activated protein kinase (AMPK) in order to regulate protein translation and to reduce food intake (Cota et al., 2006; Ropelle et al., 2008). Indeed, central injection of leucine in the ventromedial hypothalamic nucleus has an anorectic effect through activation of a hypothalamic-brainstem circuit (Cota et al., 2006; Blouet et al., 2009; Haissaguerre et al., 2014). The nature of ingested AAs is also a very important parameter. Animals reject diet imbalanced in essential AAs, and forage for food with adequate AA content (Morrison et al., 2012; Anthony and Gietzen, 2013).

In the brain, AAs sensing could also implicate membrane receptors of GPCR family including the taste heterodimer receptor family (T1R1/T1R3) (Hoon et al., 1999; Li et al., 2002; Nelson et al., 2002) and CasR receptors (Conigrave et al., 2002).

The olfactory system plays a major role in AA sensing. The most studied mechanism uses SLC transporters but some receptors might also be implicated.

Sensing Role of Amino Acids in Olfactory Structures: Molecular Hallmarks

Amino Acid Transporters Expressed in Olfactory Structures

This chapter will focus attention on selected transporters that are known to be involved in metabolic regulation and are expressed in olfactory areas: the electrogenic transporters encoded by the *slc6a15*, *slc38a2,* and *slc1a5* genes and the non-electrogenic transporters encoded by *slc7a5* (**Figure 4A**).

Electrogenic solute carrier transporters (SLC6A15, SLC38A2, SLC1A5)

At least three electrogenic AA transporters are observed in olfactory areas (**Figure 4A**). They displace AAs together with Na^+ and induce a subsequent depolarization.

Two of them, SLC6A15 and SLC38A2 transport small neutral AAs like leucine, isoleucine, and valine together with Na^+ in a 1:1 stoichiometry (Yao et al., 2000; Mackenzie and Erickson, 2004; Broer et al., 2006; Hagglund et al., 2013). SLC6A15 is present in the OB, AON, and endopiriform and piriform cortices (Inoue et al., 1996; Masson et al., 1996; Drgonova et al., 2013; Hagglund et al., 2013; Allen Institute for Brain Science, 2015). SLC38A2 mRNA is three times higher in the OB than other brain areas like the hippocampus, hypothalamus, cortex, or PC (Sundberg et al., 2008; Allen Institute for Brain Science, 2015). SLC38A2 is associated with the general amino acid control non-derepressible 2 (GCN2) pathway (Blais et al., 2003; Palii et al., 2006; Gietzen and Aja, 2012; Taylor, 2014). This pathway is activated when essential AAs are deficient

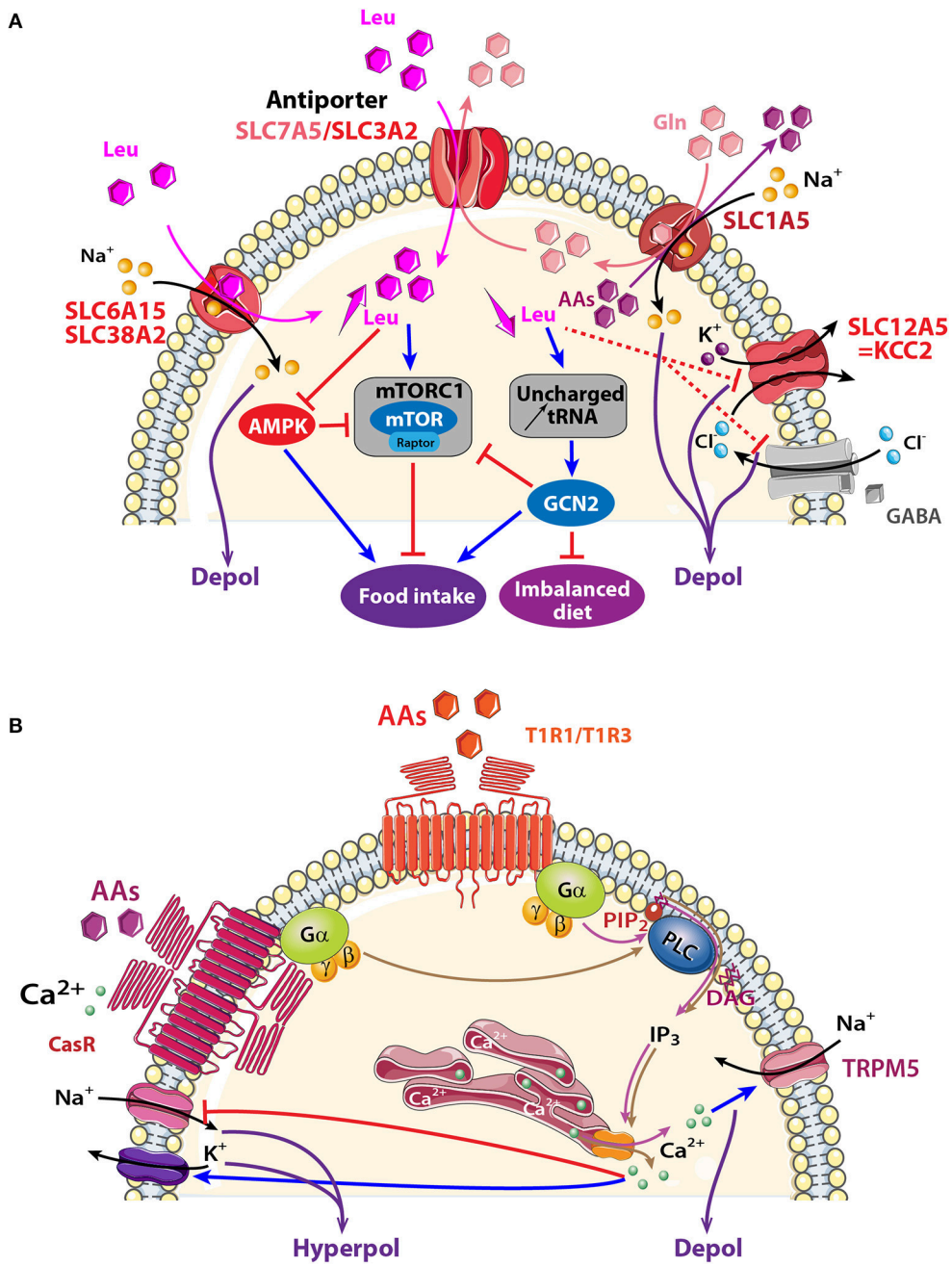

FIGURE 4 | Schematic model showing AA sensing signaling pathways that might modulate neuronal activity of central olfactory areas. **(A)** Three electrogenic transporters (SLC6A15, SLC38A2, and SLC1A5) and one non-electrogenic antiporter SLC7A5/SLC3A2 are observed in the OB and the PC. AAs fluxes depend on physiological needs, on the importance of transported AAs (essential or non-essential), and on the cellular gradient of AAs. When leucine (Leu) and glutamine (Gln) are highly available, they are co-transported with sodium inside the cell through SLC6A15, SLC38A2 or SLC1A5. Intracellular Gln is in turn co-exchanged with Leu via the bidirectional antiporter SLC7A5/SLC3A2. The anterior PC (APC) detects essential AA deficiency that increases uncharged tRNA and activates the general amino acid control non-derepressible 2 (GCN2) pathway. The concomitant down regulation of $GABA_A$ receptor and KCC2 transporter disinhibits the APC that send messages to nutritional brain areas in order to stop eating the imbalanced diet. Signaling proteins of the mammalian target of rapamycin complex1 (mTORC1) and AMP-activated protein kinase (AMPK) pathways are also present in olfactory areas, which suggests that these structures could also be implicated in detecting AA abundancy or scarcity and indirectly modulating food intake. **(B)** Two AA receptors are described: T1R1/T1R3, and CasR receptors. Both are G-protein-coupled receptors and AA binding activates heterotrimeric GTP-binding proteins composed of α-gustducin (Gα) and Gβγ subunits (brown and pink arrows). Gαpromotes phosphatidylinositol phosphate 2 (PIP_2) activation of phospholipase C (PLC), leading to the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (D). IP_3 opens ion channels on the endoplasmic reticulum, releasing Ca^{2+} into the cytosol of cells. Depending on the specific ion channels present on the membrane, a cell could be depolarized after melastatin-related transient receptor potential (TRPM5) channel opening or could be hyperpolarized after Na^+ channel closure (red line) or Ca^{2+}-dependent-K^+ channel opening (blue arrow). AAs: hexagons; activation: blue arrow, inhibition: red line. Direct and indirect action of one molecule: full and dotted line respectively.

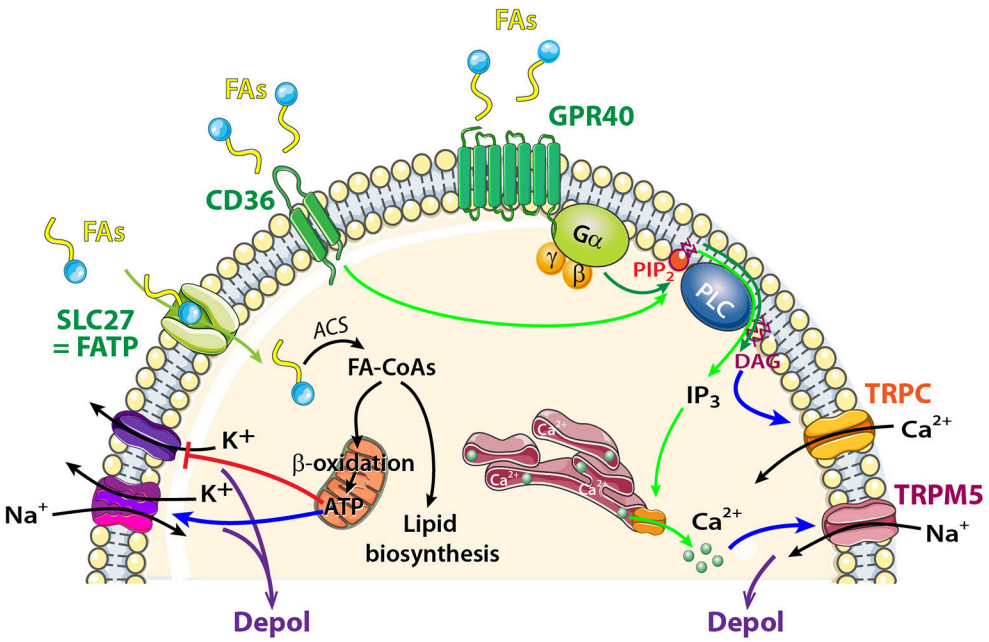

FIGURE 5 | Schematic model showing FA sensing signaling pathways that might modulate neuronal activity of central olfactory areas. The transporter SLC27 induces influx of FAs, and acyl-CoA synthetase (ACS) to esterify FAs to fatty acyl-CoAs (FA-CoAs). Following mitochondrial β oxidation of FA-CoAs, production of ATP induces depolarization by acting on a wide variety of ATP dependent ion channels. FAs Receptors: Activation of CD36 by FA binding (light green arrows) causes phosphorylation of protein tyrosine kinases, leading to generation of inositol 1,4,5-trisphosphate (IP_3) that induces Ca^{2+} release from the endoplasmic reticulum. $[Ca^{2+}]_i$increase depolarizes the membrane via TRPM5 channel. FAs receptors 7TM GPR40 receptor signaling (dark green arrows) acts through heterotrimeric G-proteins and produces IP3 and diacylglycerol (DAG). Phospholipase C (PLC) and DAG activate transient receptor potential cation channel subfamily C (TRPC).

causing accumulation of uncharged tRNA (Zhang et al., 2002; Maurin et al., 2005; Gietzen and Aja, 2012). One or two hours after AA reduction, SLC38A2 synthesis is upregulated in order to increase AA uptake (Blais et al., 2003; Palii et al., 2006; Gietzen and Aja, 2012; Taylor, 2014). Deficiency in essential AAs affects the PC where it causes downregulation of $GABA_A$ receptors and the K^+/Cl^- co-transporter (KCC2), also known as SLC12A5 (Sharp et al., 2013). KCC2 is localized in GABAergic neurons in the OB and PC (Wang et al., 2005; Sharp et al., 2013).The PC is thus identified as the central structure that detects imbalanced diet lacking essential AAs. PC activation interrupts protein synthesis in 20 min and stops food intake in animals to promote foraging for a more appropriate diet (Leung et al., 1968; Koehnle et al., 2003; Gietzen and Aja, 2012; Morrison et al., 2012).

The third transporter, SLC1A5, is an antiport that exchanges one Na^+ and glutamine against neutral AAs in a 1:1 stoichiometry (Kanai and Hediger, 2004; Nicklin et al., 2009; Pochini et al., 2014). SLC1A5 has long been considered an electroneutral transporter (Utsunomiya-Tate et al., 1996) but recently Scalise and collaborators suggested that more than one Na^+ might be transported (Scalise et al., 2014). A wide distribution of the *slc1a5* is shown in MCs and the glomerular layer of the OB, and in the PC (Allen Institute for Brain Science, 2015). Glutamine and leucine intake through SLC1A5, together with SLC7A5/SLC3A2 (described in the next section), are proposed to be upstream steps of mTORC1 activation (Nicklin et al., 2009). The presence of these transporters in olfactory structures together with molecules involved in the mTORC1 pathway, such as raptor (Bar-Peled and Sabatini, 2014; Haissaguerre et al., 2014) makes it compelling to look for looking for AAs sensing through activation of the mTORC1 pathway in the olfactory system.

Non-electrogenic solute carrier transporter (SCL7A5/SLC3A2)

SLC7A5 is associated covalently with the glycoprotein SLC3A2. Both SLC7A5 and SLC3A2 are expressed in the OB, hippocampus, and hypothalamus (Kageyama et al., 2000; Allen Institute for Brain Science, 2015). SLC7A5/SLC3A2 is an AA exchanger that combines efflux of glutamine to influx of large neutral AAs like leucine with a 1:1 stoichiometry. Intracellular AA availability limits its transport rate given the low affinity of the intracellular domain of the transporter compared with its extracellular domain (Meier et al., 2002; Verrey, 2003). The net transport of AAs through SLC7A5/SLC3A2 is linked with electrogenic AA transporters like SLC1A5 that provides intracellular AAs for SLC7A5/SLC3A2 functioning. As a consequence, a reduced influx of glutamine through electrogenic transporters could limit leucine influx through SLC7A5 and consequently block the mTORC1 pathway (Verrey, 2003; Nicklin et al., 2009; Taylor, 2014).

Amino Acid Receptors Expressed in Olfactory Structures

Taste Receptor Family (T1R1/T1R3) Expressed in Olfactory Structures

Taste buds of the tongue express the heterodimer receptor (T1R1/T1R3) belonging to a GPCR family that detects essential AAs (Hoon et al., 1999; Li et al., 2002; Nelson et al., 2002). *Tas1r1* and *Tas1r3* genes encoding for this receptor, and their associated G-proteins are found in a variety of central areas including the OB, hypothalamus and hippocampus, (Ren et al., 2009; Allen Institute for Brain Science, 2015; Voigt et al., 2015). Most members of the IP_3 transduction pathway triggered by T1R1/T1R3 activation in the taste buds and the cation channel TRPM5 (Chaudhari et al., 2009; Chaudhari and Roper, 2010) are present in the OE, OB, and PC (Ross et al., 1989; Lin et al., 2007; Rolen et al., 2014; Allen Institute for Brain Science, 2015; Pyrski et al., 2017). In the future, studying the role played by T1R1/T1R3 in olfactory areas will be interesting in the context of AAs sensing (**Figure 4B**).

Calcium Receptor Family (CasR) Expressed in Olfactory Structures

The localization and function of CasR in olfactory structures is species variant. In the OE of fish, CasR has the capacity to detect environmental Ca^{2+} and nutrients (Loretz, 2008). In rats, CasR transcript is expressed in the OB, AON and PC (Rogers et al., 1997; Ferry et al., 2000; Yano et al., 2004; Mudo et al., 2009). CasR is a multimodal receptor and it has been proposed to contribute to Ca^{2+} homeostasis and AA transport in neurons (Conigrave et al., 2002). When extracellular Ca^{2+} concentration reaches a threshold, CasR cooperatively binds to Ca^{2+} and to aromatic, aliphatic, or polar AAs (Conigrave et al., 2002; Conigrave and Hampson, 2006). Various intracellular pathways, including the downstream IP_3 pathway, are activated to release internally stored Ca^{2+} (Hofer, 2005; Zhang et al., 2015). Excitability is reduced by opening Ca^{2+}-dependent potassium channels and closing sodium channels (Han et al., 2015; Jones and Smith, 2016). The presence of CasR in olfactory structures together with components of IP_3 pathway are good cues to investigate in the future if this transport allows olfactory structures to sense AAs.

Metabolic Dysfunction and Amino Acid Sensors in Olfactory Areas

Taken together, the fact that olfactory areas express transporters, receptors and intracellular molecules implicated in the regulation of AA content, strongly suggests that the OB and PC could play an important role in AAs sensing.

When it comes to AA sensing via transporter activation, two mechanisms coexist: one involves the mTORC1/AMPK pathway that detects AA availability and the second one involves GCN2 that specifically alerts when one or more essential AAs are insufficiently ingested. The hypothalamus is proposed to be the center for mTORC1/AMPK signaling (Cota et al., 2006; Ropelle et al., 2008; Hagglund et al., 2013) while the anterior part of PC (APC) utilizes GCN2. Leung's and Gietzen's teams have collected convergent data showing that the APC is a sensor of AAs imbalanced diet. Briefly, deficiency in one essential AA induces rapid rejection of the imbalanced diet (Leung et al., 1968; Koehnle et al., 2003; Gietzen and Aja, 2012; Morrison et al., 2012). This aversion disappears after APC lesion (Leung and Rogers, 1971) and persists after hypothalamus or OB injury (Leung and Rogers, 1970; Leung et al., 1972), which identifies the APC as the sensor of an AA imbalanced diet. Moreover, local injection of the deficient AA in the APC reduces food aversion by maintaining consumption of the imbalanced diet (Beverly et al., 1990; Russell et al., 2003). Accumulation of uncharged tRNA caused by AAs deficiency activates the GCN2 pathway (Hao et al., 2005; Rudell et al., 2011) and disinhibits the APC mainly through downregulation of $GABA_A$ receptor and KCC2, also known as SLC12A5 transporter (Sharp et al., 2013). KCC2 is localized in GABAergic neurons in the OB and PC (Wang et al., 2005; Sharp et al., 2013). Glutamatergic pyramidal neurons in the APC would then send messages to feeding circuits, including the hypothalamus, in order to stop food intake (Gietzen and Magrum, 2001). Noteworthy is that mTORC1 is not involved here because behavioral rejection of the improper diet remains in the presence of rapamycin (Hao et al., 2010) (**Figure 4A**).

The role played by the APC in sensing AA deficiency is thus clear. However, sensing AA abundance via other olfactory structures has not been explored yet. It would be interesting to explore the possible implication of OB and/or PC in detecting AA abundancy and scarcity through mTORC1/AMPK pathways and through AA receptor activation.

Another sensor of AAs, *Tas1R1*, seems to be dependent on the feeding state when expressed in the hypothalamus. *Tas1r1* levels increase following a 24-h food deprivation (Ren et al., 2009). *Tas1r1* is highly expressed in the hypothalamus of obese and hyperglycemic ob/ob mice. The similarities between the nutrient sensing properties of the hypothalamus and that of the OB (**Figure 1**) prompt further investigation of the role of T1R1 or the gene it encodes *Tas1r1*, in sensing AAs in olfactory structures.

FATTY ACID SENSING

Physiological Role of Fatty Acid Supply to the Brain

The brain is roughly 50% fatty acids (FAs) by weight making it the organ with the second highest lipid content after that of adipose tissue (Watkins et al., 2001). Cerebral lipids are uptaken from the blood or synthesized locally (Rapoport et al., 2001; Smith and Nagura, 2001). Indeed, brain neurons express enzymes for both intracellular metabolism and *de novo* synthesis of FAs (Le Foll et al., 2009). In the human brain, the main source of polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid, eicosapentaenoic acid, and arachidonic acid, is dietary. Even though free FAs are not the primary metabolic fuel for neurons, they are key components of membranes and intracellular signaling pathways. PUFAs are of great importance in neurobiology because they are essential for neurogenesis, memory, learning, and play a key role in modulating ion

channels and neurotransmitter receptors. In fact, an adequate lipid environment is vital for the normal functioning of neuronal membrane proteins such as ion channels, enzymes, ion pumps, and receptors. Long-term nutritional PUFA deficiency impairs brain functioning (Khan and He, 2017). FA sensing in neurons was first reported by Oomura et al. (1975). Since then, a growing body of evidence has established the importance of brain FA sensing in the regulation of food intake (Loftus et al., 2000; Lam et al., 2005; Levin et al., 2011). Specific areas of the central nervous system including the hypothalamus, brainstem, and hippocampus (Gao and Lane, 2003; Lam et al., 2005; Picard et al., 2014) have been shown to use FAs as cellular messengers to inform "FA-sensitive neurons" about the energy status of the body (Migrenne et al., 2011). Similar to glucose sensing and AAs sensing described previously, lipid sensing is involved in the control of feeding behavior (Obici and Rossetti, 2003; Cruciani-Guglielmacci et al., 2004). Hypothalamic lipid sensing mechanisms are disrupted during conditions of prolonged fasting (Yue and Lam, 2012). The molecular mechanisms involved in FA sensing by the brain are still a matter of debate.

The FA transporter proteins (FATP also called SLC27), is a protein family of six isoforms. SLC27A4 (FATP4) is the major FATP expressed in the brain (Fitscher et al., 1998). In hypothalamic neurons, FAs are transported inside cells through FATPs. FAs are then oxidized to generate ATP that can modulate the activity of a wide variety of ATP-dependent ion channels including K^+ channels, and the Na^+-K^+ ATPase pump. The resulting change in neuronal firing rate suggests that FAs metabolism play a role in the regulation of energy balance (Migrenne et al., 2011; Picard et al., 2014).

In the brain, membrane receptors mediating FAs sensing consist of two GPCRs (GPR40 and GPR120) and CD36, often associated to fatty acid translocase (FAT) to make a translocator/receptor complex FAT/CD36. CD36 has been reported to be involved in FA sensing in taste buds (Fukuwatari et al., 1997; Laugerette et al., 2005) and in hypothalamic neurons (Le Foll et al., 2009). Hypothalamic CD36 expression induced by fasting or following high-fat diet, could modulate lipid signaling in the brain and participate in the regulation of energy homeostasis (Moulle et al., 2012, 2014). All together, these findings strongly suggest that lipid sensing by CD36 is responsible for basic physiological functions in relation to behavior and energy balance (Martin et al., 2011). In the hypothalamus, it has been postulated that binding of FAs to CD36 alters neuronal activity in a manner analogous to that utilized for fat perception by taste receptor cells (Le Foll et al., 2009). This causes phosphorylation of protein tyrosine kinases, leading to generation of IP_3, recruitment

TABLE 1 | Overview of nutrient sensing molecular cues and their corresponding nutrients, present in olfactory structures.

Nutrient	Nutrient sensing cues	Olfactory areas	References
Glucose	GLUT3	OE, OB	Vannucci et al., 1998; Nunez-Parra et al., 2011
	GLUT4/IR	OB, AON, PC, OT	Leloup et al., 1996; El Messari et al., 1998, 2002; Vannucci et al., 1998; Choeiri et al., 2002; Aimé et al., 2014; Al Koborssy et al., 2014; Kovach et al., 2016
	SGLT1	OB	Aimé et al., 2014; Al Koborssy et al., 2014
	Kv1.3	OB	Tucker et al., 2010, 2013; Kovach et al., 2016
	mTORC1	OB, PC	Allen Institute for Brain Science, 2015
Amino acid	SLC7A5/SLC3A2	OB	Kageyama et al., 2000; Allen Institute for Brain Science, 2015
	SLC1A5	OB	Allen Institute for Brain Science, 2015
	SLC6A5	OB, AON, PC	Inoue et al., 1996; Masson et al., 1996; Drgonova et al., 2013; Hagglund et al., 2013
	SLC38A2	OB, PC	Sundberg et al., 2008; Allen Institute for Brain Science, 2015
	KCC2	PC	Wang et al., 2005; Sharp et al., 2013
	GCN2	PC	Maurin et al., 2005; Anthony and Gietzen, 2013
	mTORC1	OB, PC	Allen Institute for Brain Science, 2015
	T1R1	OB	Allen Institute for Brain Science, 2015; Voigt et al., 2015
	T1R3	OB	Allen Institute for Brain Science, 2015; Voigt et al., 2015
	TRPM5	OE, OB, PC	Lin et al., 2007; Rolen et al., 2014; Allen Institute for Brain Science, 2015; Pyrski et al., 2017
	GPCRs type CasR	OE	Loretz, 2008
		OB, AON, PC	Rogers et al., 1997; Ferry et al., 2000; Yano et al., 2004; Mudo et al., 2009
Fatty acid	SLC27A1, SLC27A4	OB, AON, PC	Allen Institute for Brain Science, 2015
	mTORC1	OB, PC	Allen Institute for Brain Science, 2015
	GPR40 (FFA1)	OB	Nakamoto et al., 2012; Khan and He, 2017
	CD36	OE, OB	Benton et al., 2007; Lee et al., 2015; Oberland et al., 2015
	TRPC	OB	Otsuka et al., 1998; Philipp et al., 1998; Dong et al., 2012
	TRPM5	OE, OB, PC	Lin et al., 2007; Rolen et al., 2014; Allen Institute for Brain Science, 2015; Pyrski et al., 2017

of Ca^{2+} from the endoplasmic reticulum, followed by influx of calcium via opening of store-operated calcium channels, membrane depolarization via TRPM5 channel activation, and ultimately neurotransmitter release (El Yassimi et al., 2008).

In this review, only FA transporters (FATP/SLC27) and the FA receptors GPR40 and CD36 will be detailed. Intracellular proteins including long-chain fatty acyl-coenzyme A (CoA) synthetases and FA oxidative proteins are largely involved in neuronal FA sensing but are beyond the scope of this review (Picard et al., 2014).

Sensing Role of Fatty Acids in Olfactory Structures: Molecular Hallmarks

Fatty Acid Solute Carrier Transporters Expressed in Olfactory Structures (SLC27)

According to the Allen Mouse Brain Atlas, SLC27A1 and SLC27A4 are expressed in the OB, AON, and PC. In the OB, SLC27A4 is mainly expressed in MCs (Allen Institute for Brain Science, 2015). While no previous study has investigated lipid sensing in central olfactory structures, many molecular cues seem to suggest that free FAs could be used as a messenger in these olfactory areas neurons to inform about the energy status of the whole body (**Figure 5**).

Fatty Acid Receptors Expressed in Olfactory Structures

GPR40 (but not GPR120) is highly expressed in the OB (Nakamoto et al., 2012; Khan and He, 2017). Like all GPCRs, GPR40 is coupled to an intracellular heterotrimeric G protein (Gα) that activates the phospholipase C (PLC) located on the plasma membrane. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into 2 s messengers: IP_3 and diacyglycerol (DAG) (**Figure 5**). The generation of PLC facilitates transport of PKC from the cytosol to the plasma membrane. PLC, PKC, and DAG were described as activators of the TRP subfamily C (Khan and He, 2017). In the OB, MCs and external tufted cells extensively express TRPC3, C4, and C5 whereas neurons of the granule cell layer express TRPC1 and C4 only (Otsuka et al., 1998; Philipp et al., 1998; Dong et al., 2012). Studying modulation in MCs firing in response to fluctuations in extracellular FA concentration would be interesting in the context food intake and/or food choice.

In addition to GPRs, CD36 is a well described receptor for FAs. In the peripheral olfactory system, CD36 has been identified in insect and rodent OSNs (Benton et al., 2007; Lee et al., 2015). In recent studies, CD36 has been localized in the cilia, dendrites, and soma of a subset of OSNs in young rodents (Lee et al., 2015; Oberland et al., 2015). The CD36-positive OSNs respond in an age-dependent manner to oleic acid, a major milk component. This suggests that CD36 is involved in FA detection by the peripheral olfactory system during the suckling period (Oberland et al., 2015). CD36 was also found in central olfactory areas such as the glomerular layer of the OB (Oberland et al., 2015), PC and nucleus of the lateral olfactory tract (Glezer et al., 2009). The role of CD36 in these central olfactory areas has been raised whereby similar to taste buds, CD36 would sense FAs. TRPM5 channel is present in the OE, OB, and PC (Lin et al., 2007; Rolen et al., 2014; Allen Institute for Brain Science, 2015; Pyrski et al., 2017) and can serve as a downstream member of FA sensing where it is activated by an increase in Ca^{2+}; the latter resulting from FA intake. CD36 activation would be investigated in the context of FAs sensing of olfactory areas.

Metabolic Dysfunction and Lipid Sensors in Olfactory Areas

In contrast to glucose and AAs sensing, only one study has explored the neuron lipid sensing in peripheral olfactory structures (Oberland et al., 2015). The fact that CD36, GPR40 and molecules involved in their intracellular pathways, are expressed in neurons of olfactory structures raises the question of their role(s) in lipid olfactory perception, central FA sensing, and regulation of energy balance. Indeed, lipid sensing is described as an important contributor to the regulation of energy balance (Magnan et al., 2015). In circumvallate taste buds, a decrease in CD36 expression induced by high-fat diet causes obesity and reduced sensitivity to fat taste, which in turn increased the intake of fatty foods as a compensatory response (Zhang et al., 2011). In the same way, reduction in hypothalamic CD36 expression induced redistribution of fat from visceral to subcutaneous deposits and markedly impaired insulin sensitivity (Le Foll et al., 2009, 2013, 2015). Growing evidence shows that dysregulation of brain FA sensing may contribute to energy imbalance and development of obesity, associated with type 2 diabetes or not (Yue and Lam, 2012; Picard et al., 2014). It will be interesting in future studies to investigate if olfactory dysfunction caused by altered energy balance (Thiebaud et al., 2014) could be linked to a change in expression of GPR40 and/or CD36.

CONCLUSION

In order to regulate nutrient homeostasis, the body initiates multiple and redundant mechanisms in response to modulation in internal nutrient levels. In addition to the hypothalamic regulatory center, olfactory structures are proposed to detect both odors and nutrients. In this manner, the olfactory system contributes, through foraging and food, selection in maintaining metabolic homeostasis. In particular, mounting evidence indicates that the OB and the PC are involved in food intake, via regulation of choice of food with the appropriate nutrient content. This review presents a new approach to the problem of energy balance by suggesting that the nature of ingested nutrients could act on subpopulations of nutrient sensing neurons discreetly located in key brain areas including olfactory areas. In spite of numerous arguments described in this review (see **Table 1**), our understanding of the mechanisms implicated in nutrient sensing in olfactory areas is far from complete. The links between hormones involved in food intake regulation and that of nutrient sensing have to be deciphered. In the hypothalamus the mTORC1 is known to be a key component of the intracellular path integrating all these

internal signals (i.e., nutrients and hormones) (Wullschleger et al., 2006; Wiczer and Thomas, 2010; Haissaguerre et al., 2014). We suggest that nutrient sensing in olfactory areas, could involve mTORC1 signaling. However, GCN2, and not mTORC1, is necessary for the detection of AA imbalance in the PC (Hao et al., 2010). The role of mTORC1 in detecting over consumption of nutrients in the PC, is a separate question to investigate. In addition to these and other unanswered questions, we still lack an integrative view of the presumably coordinated role played by olfactory areas and the hypothalamus regarding their metabolic homeostasis. Deciphering these aspects might offer new solutions in mitigating metabolic dysfunctions such as obesity and/or diabetes and provide new approaches to investigate physiological functions such as memory, and sleep that exhibit reciprocal relationships with homeostasis regulation and olfactory function (Barnes and Wilson, 2014).

AUTHOR CONTRIBUTIONS

AJ and BP were responsible for the conception and design of the review; DK, BP, and AJ drafted the review; All authors revised the manuscript critically for important intellectual content and approved the final version of the manuscript.

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REVIEW
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Emerging Molecular Pathways Governing Dietary Regulation of Neural Stem Cells during Aging

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Aging alters cellular and molecular processes, including those of stem cells biology. In particular, changes in neural stem cells (NSCs) are linked to cognitive decline associated with aging. Recently, the systemic environment has been shown to alter both NSCs regulation and age-related cognitive decline. Interestingly, a well-documented and naturally occurring way of altering the composition of the systemic environment is through diet and nutrition. Furthermore, it is well established that the presence of specific nutrients as well as the overall increase or reduction of calorie intake can modulate conserved molecular pathways and respectively reduce or increase lifespan. In this review, we examine these pathways in relation to their function on NSCs and cognitive aging. We highlight the importance of the Sirtuin, mTOR and Insulin/Insulin like growth factor-1 pathways as well as the significant role played by epigenetics in the dietary regulation of NSCs and the need for further research to exploit nutrition as a mode of intervention to regulate NSCs aging.

Keywords: nutrients, diet, aging, mTOR, sirtuins, insulin-like growth factor signaling, IIS, epigenetics

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AGING AND NEURAL STEM CELL FUNCTION

Aging is the number one risk factor for the majority of diseases currently affecting the developed and developing world (Niccoli and Partridge, 2012). The area of study addressing this issue, known as biogerontology, is committed to investigating the underlying mechanisms of aging, to explore whether they can be intervened upon to delay or perhaps even halt the progression of age-related conditions such as cardiovascular disease, cancer and neurodegeneration (Verburgh, 2015). An improved understanding of aging mechanisms could lead to the development of strategies to increase "healthspan"—the period of time free from debilitating disease (Franklin and Tate, 2008; Brandhorst et al., 2015). Aging is linked to a number of cellular and molecular processes including nutrient-sensing pathway, epigenetic and stem cells biology deregulation (López-Otín et al., 2013). Stem cells in general have been closely linked to aging owing to their reduced regenerative ability linked to the decline of tissues that accompanies age (Signer and Morrison, 2013; Behrens et al., 2014). Impaired function of satellite stem cells in muscle and epidermal stem cells of the skin, for example, is a key process underlying reduced regeneration during aging in these tissues (Castilho et al., 2009; Day et al., 2010).

The adult NSC population is also negatively affected by age. Post-natal NSCs, able to differentiate into neurons, have been identified in several areas of the mammalian central nervous system (CNS), including the rodent and human dentate gyrus (DG) of the hippocampus

and the mouse subventricular zone (SVZ). Postnatal-born neurons—a phenomenon known as adult neurogenesis—in these areas have been implicated in learning and memory and olfaction respectively (Altman and Das, 1965; Pencea et al., 2001; Spalding et al., 2013). More recently post-natal NSC-derived neurons have also been identified in the mouse hypothalamus and the human striatum (Kokoeva et al., 2005; Ernst et al., 2014). Though mouse models have been extensively used in adult neurogenesis research, it is becoming clear that there are considerable differences between rodent and human neurogenesis: firstly, there seems to be no neuron turnover in the human olfactory bulb, whilst this is an important neurogenic zone in the mouse brain. Secondly, whilst hippocampal neurogenesis occurs in both species, retrospective birth dating of human postmortem tissue has revealed that nearly all of the dentate granule "turnover" during adult life, whereas only 10% of the mouse granule neurons are exchanged in adulthood (Spalding et al., 2013). Furthermore, the rate of decline in hippocampal neurogenesis in response to age appears far greater in mice than in humans (Bergmann et al., 2015).

The marked decline in NSC activity in the aging rodent DG manifests in reduced proliferation that eventually leads to the depletion of the progenitor pool (Romine et al., 2015; Yang et al., 2015). This decline partly contributes to the age-linked decline in cognitive abilities, particularly those dependent on the hippocampus (Park et al., 2013; Romine et al., 2015; Yang et al., 2015). In the SVZ, there is a similar marked decline in NSC function and this contributes to impoverished olfaction during rodent aging (Enwere et al., 2004). Studies in model species have shown that deregulated hippocampal neurogenesis, is an important component of neurodegenerative conditions such as Alzheimer's (López-Toledano and Shelanski, 2004, 2007; Winner et al., 2011), further linking declines in NSC function to the deterioration of the aging brain. Aging therefore appears to exert some of its detrimental effects in the CNS by directly interfering with multiple cellular and molecular processes that govern the regulation of the NSC population. This notion is summarized in **Figure 1**. As aging has such severe effects on the decline of NSC and the development of neurodegenerative conditions, this prompts the field to consider whether we can we target the maintenance of the NSC population to slow cognitive aging and neurodegenerative disease progression? If so, what are the key mechanisms to target?

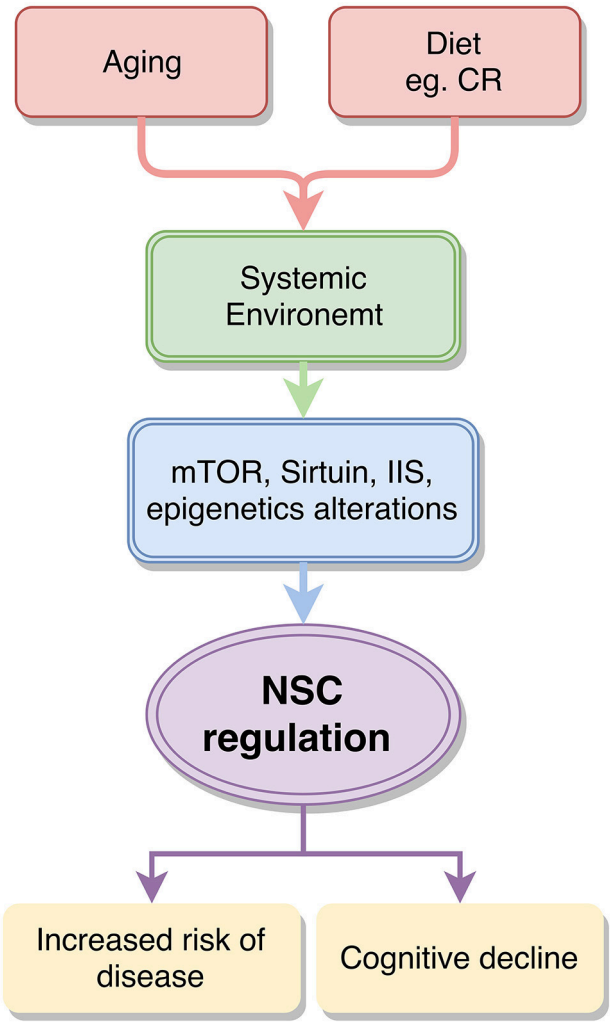

FIGURE 1 | Diagram summarizing the proposed relationship between aging, nutrition and neural stem cells. Aging and dietary modulation such as calorie restriction (CR) can alter the composition of the systemic environment, which affects epigenetic regulation and several nutrient-sensing pathways, including the mTOR, Sirtuin and Insulin and Insulin like signaling (IIS) pathways. These in turn alter neural stem cell (NSC) regulation and can predispose to disease and cognitive decline.

Interestingly, studies investigating the role of aging on NSCs have also highlighted the importance of the systemic milieu in regulating the neurogenic niches of the CNS; through the use of heterochronic parabiosis, Villeda and colleagues showed that the systemic environment of a young mouse was able to rescue the cognitive deficits of an aged mouse following the fusion of young-old circulatory systems (Villeda et al., 2011, 2014). This rejuvenating effect is also observed following intravenous infusion of young plasma into old mice, and was underpinned by a marked reversal of age-related decline in hippocampal neurogenesis (Villeda et al., 2011) and upregulation of genes linked to synaptic plasticity (Villeda et al., 2014). Similarly, enhanced neurogenesis in the SVZ was observed by Katsimpardi and colleagues following heterochronic parabiosis, resulting in improved odor discrimination (Katsimpardi et al., 2014). Notably, these studies have demonstrated the role for candidate chemokines (e.g., CCL11, Villeda et al., 2011) and growth factors (e.g., GDF11 e.g., Loffredo et al., 2013) whose circulating levels fluctuate in aging mice and appear to exert their effects, at least in part, by altering NSC function. Complementary to this, studies looking at other populations of stem cells have observed a similarly rejuvenating effect of the youthful milieu on the typical age-related declines in stem cell function throughout the body as well as vice-versa, whereby the old milieu inhibited stem cell function (Conboy and Rando, 2012). These data suggest that by altering the composition of the systemic environment, one could affect the regulation of the NSCs; a well-documented, naturally occurring way of achieving

this is through diet and nutrition (Stangl and Thuret, 2009). We define diet as encompassing dietary paradigms such as calorie restriction (CR), intermitted fasting (IF), dietary restriction (e.g., of specific components such as protein) (DR) and time restricted feeding (TRF) whereas nutrition involves the intake and/or supplementation of foods containing pro-neurogenic agents such as polyphenols, polyunsaturated fatty acids (PUFAs) and vitamins/minerals.

As stem cells in general are designed to act in response to their environment and, and as reported above, are responsive to differing compositions of the systemic environment, it follows that diet can greatly influence their function (Murphy and Thuret, 2015). Several authors have reviewed the effect of diet on stem cells in general (Rafalski et al., 2012; Ochocki and Simon, 2013; Mihaylova et al., 2014), for the purpose of this review we will focus on the relatively unexplored field of the effects of diet and aging on NSCs. Briefly, nutritionists have found that an overabundance of nutrients is detrimental to several aspects of human and animal health (Keenan et al., 1994; Nagai et al., 2012). This results from an overstimulation of nutrient sensing molecular pathways, which eventually become insensitive to the stimuli (Blagosklonny, 2008; Gems and de la Guardia, 2013). CR, IF and DR have been reported to have opposite effects on these pathways and present a means to improved health and life span (Solon-Biet et al., 2014, 2015; Brandhorst et al., 2015; Fontana and Partridge, 2015). **Figure 1** is a schematic of how aging, diet and the systemic environment interact to act upon these pathways. Notably, these effects are conserved across species as they have been observed in simple organisms such as yeast, *C. elegans*, and drosophila through to rodents and humans (Fontana et al., 2010). We now review the impacts of dietary and nutritional regulation of NSC activity and function, with focus on the aforementioned nutrient sensing pathways and their effects on longevity.

IMPACT OF DIET ON NEURAL STEM CELLS

Dietary paradigms such as CR and IF are the most widely employed means of assessing the impacts of diet upon stem cell function and longevity. A 30–40% reduction in calorie intake, the typical regimen for CR, has often been brought about by alternate-day feeding—a form of IF. As such, the field must pay great attention as to whether they are assessing the impacts of DR or IF, unpublished work from our lab shows that the positive impacts of IF on hippocampal neurogenesis and cognitive performance can be derived independent of calorie intake, suggesting that it is the period of fasting that that is acting on the NSC pool. Nutritional content, such as the amount of polyphenols and PUFAs, among other specific nutrients, has also been reported to impact on NSC function during aging (see Maruszak et al., 2014; Murphy and Thuret, 2015 for review).

Reducing calorie intake in rodents was shown to counteract age-related cognitive decline as well as increase the number of newly generated neurons in the hippocampus (Ingram et al., 1987; Lee et al., 2000, 2002). Furthermore, Kumar and colleagues suggest DR may aid in fighting excitotoxic injury as an increase in progenitors is seen in the SVZ, SGL, hypothalamus and cortex of adult rats (Kumar et al., 2009). In contrast, Bondolfi and colleagues found that CR did not affect the rate of neurogenesis but affected the survival of new–born glia in the mouse hippocampus (Bondolfi et al., 2004). Given that CR and IF (typically involving some degree of CR) have been shown to positively impact on NSC function, it follows that excessive calorie intake, as in the case of obesity and other models of metabolic disorders, will negatively affect NSC activity and may decrease adult hippocampal neurogenesis (Stangl and Thuret, 2009). Furthermore, obesity was a detrimental factor in studies investigating post-stroke recovery in humans, suggesting that a history of increased calorie intake impairs brain repair (Kalichman et al., 2007). Interestingly, CR was also proven beneficial in elderly humans as shown by improved verbal memory scores (Witte et al., 2009). It must be considered however, that in elderly, sometimes frail individuals, restricting of calories may be too dangerous, older populations may therefore benefit from more targeted pharmacological interventions to modulate NSCs activity based on CR/IF and DR mimetics.

Though it may seem counter intuitive that reducing calories may be beneficial to stem cells in particular, this can be explained by our historical food supply not being readily available and abundant at all times. Humans have evolved to cope with periods of reduced calorie intake, resembling the effect achieved by CR and IF experiments. A possible biological explanation for this relies on the benefits of refeeding after a fasting period, suggesting that when an organism is "fasting" it can focus on preparing resources to act quickly and effectively when nutrients do become available (Reed et al., 1996). With respects to the NSCs of the DG in particular, their activity is possibly enhanced in the absence of nutrients owing to the necessity of "hunting behavior" and the requirement for cognitive flexibility that must accompany it: improved cognition may be a means to ensure food is found (As discussed by Mattson, 2012).

SUPPORTING EVIDENCE FOR THE ROLE OF NUTRIENT-SENSING PATHWAYS IN NEURAL STEM CELL REGULATION AND LONGEVITY

Owing to the compelling research relating diet to longevity and to NSCs, the field is now trying to delineate the molecular pathways behind this relationship. Though many pathways are involved in NSC regulation and an equally vast amount is involved in nutrient-sensing and aging, there is a relatively small proportion identified as relating the three. Thus far, the best characterized, and therefore most promising starting point for imminent studies, are the mTOR, Insulin and Insulin-like growth factor signaling and Sirtuin pathways. In this section, we will focus on the available data supporting a role for these pathways in aging, nutrient sensing and NSC regulation. See **Figure 2** for a summary of key NSC related functions affected by these pathways.

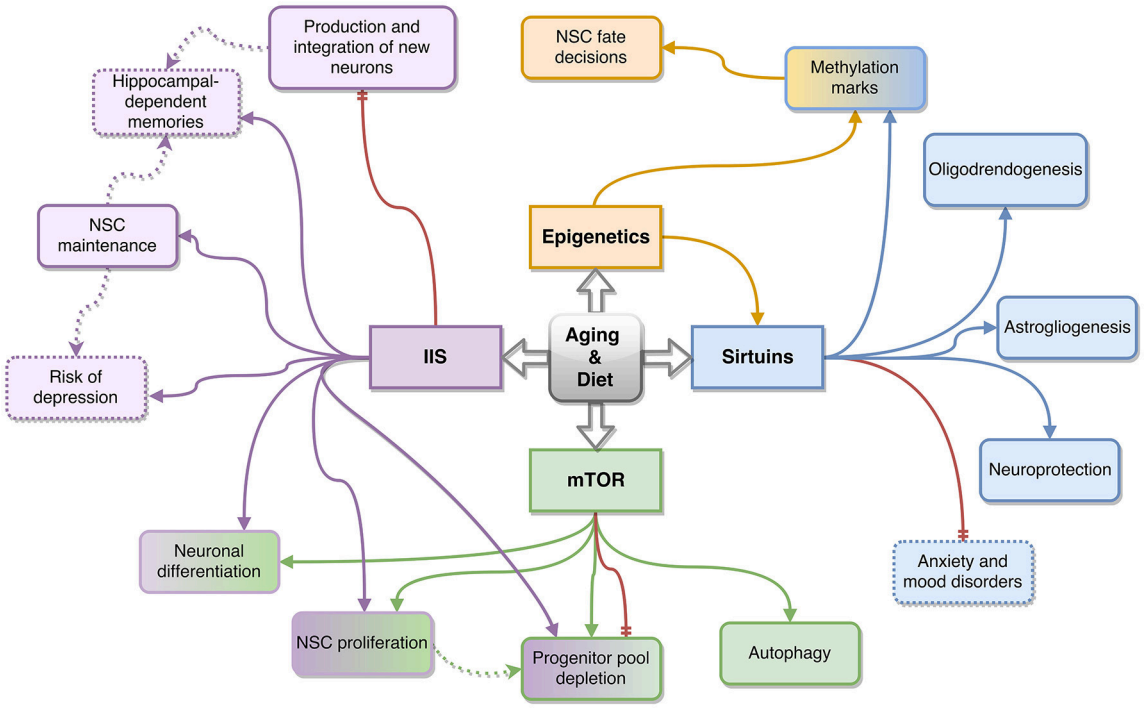

FIGURE 2 | Schematic summarizing the key effects of the Insulin/Insulin-like Growth factor (IIS) (in purple), mTOR (in green), sirtuin (in blue) and epigenetic (in orange) pathways on neural stem cell (NSC) function as a result of diet and aging. Solid arrows represent relationships between the molecular pathways (represented by the four rectangles in the center) and NSC function (represented by the outer, rounded rectangles) or related phenotypes (dotted rounded rectangles) discussed in this review. The dotted arrows connect possible molecular mechanisms behind the observed phenotypes reported in this review. Red arrows represent an effect caused by the inhibition of the pathway they stem from. Boxes with gradient coloring represent functions affected by multiple pathways.

mTOR

The mammalian target of rapamycin (mTOR) is a classical dietary and nutrient sensing pathway. mTOR is a serine/threonine kinase. It combines with several accessory proteins including RAPTOR or RICTOR to form mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), respectively. The two complexes carry out different functions with mTORC1 being responsible for cell growth and metabolism while mTORC2 regulates cytoskeleton organization. Importantly, mTOR is the catalytic subunit of the complex and is inhibited by the presence of rapamycin, this inhibition however only occurs when mTOR is bound to the RAPTOR protein (Magri and Galli, 2013). mTOR activity is altered by energy and amino acid availability as well as growth factors, making it a key molecule within nutrition studies (Magri and Galli, 2013). mTOR is also involved in a feedback loop with insulin where insulin (discussed below) activates mTOR and mTOR phosphorylates the S6 kinase, which in turn inhibits insulin signaling (Blagosklonny, 2008). In the liver, branched chain amino acids (BCAA) in particular signal for mTOR activation, showing that protein intake and specific amino acids alter the total activation of mTOR (measured as phosphorylated mTOR / total mTOR) and ultimately longevity (Solon-Biet et al., 2014).

As well as functions in nutrient sensing, the mTOR pathway has also been linked to longevity (Blagosklonny, 2010). Its inhibition via ethylaxanthine and rapamycin was shown to improve longevity in yeast (Wanke et al., 2008) and mice (Harrison et al., 2009; Fok et al., 2014) respectively, leading to plans to test the compound in larger and longer-lived mammals such as companion dogs (Check Hayden, 2014). Recently, the mechanism of action behind rapamycin's role in longevity was suggested to involve a decrease in reactive oxygen species, as shown by experiments in the rodent liver (Martínez-Cisuelo et al., 2016). Notably, rapamycin treatment was shown to exert its beneficial effects even if started at advanced age, a factor, which usually greatly impairs the efficacy of life-extending interventions (Harrison et al., 2009). In addition, Tan and colleagues highlighted increased PI3K/Akt/mTOR pathway activation during aging as the molecular mechanism responsible for replicative senescence in vascular smooth muscle cells (Tan et al., 2016). This pathway has been the focus of aging research and is shared by both the mTOR and the insulin-like growth factor signaling (IIS) pathways as explained in Section Insulin. Several consequences of mTOR activation increase the risk of premature aging and disease, these include decreased autophagy accompanied by an increase in protein production eventually causing an increase in protein agglomeration as well as an increase in inflammation (Verburgh, 2015). Furthermore, chronic mTOR activation causes increased proliferation of several types of stem cells eventually leading to progenitor pool

depletion. Adequate mTOR regulation could thus be key in maintaining these populations throughout aging (Sato et al., 2010; Paliouras et al., 2012).

Though there is a limited number of studies investigating the role of mTOR within the NSC population, there have been several encouraging findings supporting key functions for this nutrient sensing pathway in NSC regulation and aging.

Firstly, both protein deposition and inflammation are key hallmarks of neurodegenerative conditions suggesting that mTOR hyperactivity may contribute to disease progression within the CNS (O' Neill, 2013). These observations are consistent with the evidence supporting aging as a consequence of overstimulation of signaling pathways, driven by the overabundance of nutrients. Furthermore, mTOR has also been shown to be key in dictating the proliferation rate of the adult SVZ NSC population; its inhibition in fact, caused progenitor pool depletion (Paliouras et al., 2012). In addition, Han and colleagues have also reported that, during embryonic development, mTOR carries an important role for neuronal differentiation: enhancing mTOR activity via insulin caused an increase in the number of neurons, which was counteracted by rapamycin. Intriguingly, in the presence of rapamycin the decrease in neuronal numbers was attributed to increased autophagy rather than apoptosis (Han et al., 2008). These findings also suggested the possibility of similar mechanisms taking place in adult NSCs. Indeed, Yu and colleagues showed that increased autophagic death also occurred in adult hippocampal NSC following insulin withdrawal and was accompanied by a decrease in cell density, these effects were also exacerbated by the presence of rapamycin, implicating mTOR activity (Yu et al., 2008). The authors state that in an aged environment there is decreased insulin signaling and thus increased autophagy, which may reduce the survival of stem cells. Contrary to this, the authors remark that autophagy can enhance cell survival, given its beneficial effect in clearing unwanted and malfunctioning cells, an essential mechanism in the context of aging (Cuervo, 2008). Elevated autophagy in response to reduced insulin signaling may thus not be a solely negative effect but further research is required to better delineate the modulatory role of mTOR activity in NSC during aging (Gems and de la Guardia, 2013).

The limited number of studies (See **Table 1**) however, highlights the need for further investigations into the exact mechanisms of mTOR regulation in adult NSCs, with the ultimate aim of determining whether modulation of this pathway can bring preserve the NSC pool during aging. The key effects of the mTOR pathway on NSCs elucidated this far are depicted in **Figure 2**.

Insulin

Insulin and Insulin-like growth factor (IGF) are two hormones closely linked to nutrition, as they respond to increased glucose. Insulin is released by the β cells of the pancreas and acts upon transmembrane tyrosine kinase receptor to activate downstream signaling pathways and cause glucose uptake by liver and muscle cells (van Heemst, 2010). Other members of the IGF family are under the control of growth hormone (GH) release by the pituitary gland. They also act upon tyrosine kinase receptors and activate downstream pathways that are often shared with insulin, coining the term insulin/insulin-like growth factor signaling (IIS) pathway. Stimulation of the IIS pathway results in the activation of the PI3K/Akt pathway, a pathway shared with mTOR signaling, which ultimately leads to the inactivation of the FoxO transcription factors (van Heemst, 2010). While both insulin and IGF respond to carbohydrate presence, they usually carry out slightly different roles with insulin being primarily occupied with glucose metabolism and IGF with growth and survival (Rafalski and Brunet, 2011). Besides being activated by carbohydrates such as glucose, the IIS pathway is also stimulated by proteins and is involved in a feedback loop with mTOR as described in Section mTOR (Blagosklonny, 2008).

IIS is also extensively implicated in longevity (Kimura et al., 1997; Bartke et al., 2013); overactivation, like for mTOR, leads to decreases autophagy and ultimately to the shortening of lifespan (Verburgh, 2015). The decrease of the IIS pathway in C. elegans by Kimura and colleagues was one of the first experiments to show an increase in lifespan relating to diminished insulin signaling (Kimura et al., 1997). This was confirmed by several other studies, with Blüher and colleagues showing that the knock-out of a fat specific insulin receptor resulted in an increase in lifespan of 18% (Blüher et al., 2003) and Taguchi et al finding that the knock-out of downstream substrates of the IIS pathway caused a 32% increase in female mice lifespan (Taguchi et al., 2007). Furthermore, serum IGF-1 levels in 31 different mouse strains negatively correlated to average lifespan (Yuan et al., 2009). Some encouraging evidence has also been found relating IIS to human longevity; IIS related polymorphisms correlate to lifespan (Bonafè et al., 2003; Kojima et al., 2004) and several centenarians were found to have loss of function mutations in the IGF-1 receptors (Suh et al., 2008). Conversely, people affected by acromegaly, characterized by increased GH release, experienced a 2–3 fold increase in death rate (Clayton, 2003; Suh et al., 2008; Verburgh, 2015). More recently, reduced growth hormone secretion, and thus indirectly IIS activity, was shown to correlate with human familial longevity (van der Spoel et al., 2016) and genomewide meta-analysis studies linked several gene loci to longevity and to levels of circulating IGF-related proteins (Teumer et al., 2016). These studies show that this pathway and its role in lifespan is conserved in more complex organisms.

Several rodent studies have suggested insulin and IGF may link nutrition to organism longevity through key functions in tissue-specific stem cell maintenance. Mechanisms such as autophagy, compromised by IIS overstimulation, carry out key functions within the stem cell population. FoxO3A was proven essential for efficient clearing of age-related cellular debris which is known to prevent malfunctioning of stem cells and to lead to improved longevity (Cuervo, 2008; Warr et al., 2013). The IIS pathway also appears to have important functions on the regulation of neural stem cells specifically, with studies implicating it in both development and adulthood.

IGF-1 overexpression alone and IGF-1 and insulin overexpression on embryonic NSC for example, highlighted that insulin pushes toward increased differentiation while IGF-1 pushes toward a proliferative phenotype (Arsenijevic et al., 2001). Furthermore, studies have shown that overexpression of

TABLE 1 | Table summarizing studies showing supporting evidence for the role of mTOR, IIS, and Sirtuin pathways in NSCs function.

Pathway	Model	NSC	Intervention	Supporting evidence	Study
mTOR/IIS	Rat *In vitro*	Embryonic	Increased insulin levels	Increase in differentiated neurons, which was counteracted by rapamycin	Han et al., 2008
mTOR/IIS	Rat *In vitro*	DG	Insulin withdrawal	Increased neuronal death, exacerbated by rapamycin	Yu et al., 2008
IIS	Mouse *In vitro*	SVZ and DG	*FoxO3* knockout	Decreased number of NSC and self-renewal ability	Renault et al., 2009
mTOR/IIS	Rat *In vivo*	Cortex	Treated with EGCG + TBI	Reduced NSC cell death around damaged area	Itoh et al., 2012
IIS	Rat *In vitro*	DG	Increased IGF-1	Decreased differentiation and increased proliferation of NSCs	Åberg et al., 2003
IIS	Mouse *In vitro*	Striatal	Increased insulin	Increases NSCs differentiation	Arsenijevic et al., 2001
IIS	Mouse *In vivo*	Perinatal	IGF-1 overexpression	Increase in number of neurons and of oligodendrocytes.	Carson et al., 1993
IIS	Mouse *In vivo*	Perinatal	*IGF-1* KO	Decreased proliferation and differentiation of oligodendrocytes	Ye et al., 2002
IGF-1	Mouse *In vivo*	SVZ	IGF-1R KO	Reduced age related depletion of NSC	Chaker et al., 2015
IGF-II	Mouse *In vitro*	Perinatal	IGF-II treatment	Increased NSC expansion and promoted self-renewal	Ziegler et al., 2012
IGF-II	Mouse *In vitro*	DG	Sh-RNA knockdown of *IGF-II*	Impaired proliferation	Bracko et al., 2012
Sirtuins	Mouse *In vitro*	Perinatal SVZ	Oxidation or Sirt1 activation	Enhanced astrocytic lineage	Prozorovski et al., 2008
Sirtuins	Mouse *In vitro*	Perinatal SVZ	Reducing environment	Enhanced neuronal lineage	Prozorovski et al., 2008
Sirtuins	Mouse *In vitro*	Perinatal SVZ	Sh-RNA knockdown of *Sirt1*	Disengaged neural fate from redox conditions	Prozorovski et al., 2008
Sirtuins	Mouse *In vivo*	SVZ and DG	Inactivation of *Sirt1*	Increased oligodendrocyte differentiation and myelination	Rafalski et al., 2013
Sirtuins/NAMPT	Mouse *In vivo*	DG	Measuring /ablating NAMPT	NAMPT levels decrease with age, its ablation reduces NSC proliferation and oligodendrogenesis	Stein and Imai, 2014
Epigenetics	Mouse *In vitro*	Embryonic	*Dnmt1* knockout	Increased astrocytic differentiation	Fan et al., 2005
Epigenetics	Mouse *In vitro*	Perinatal SVZ	*Dnmt3* knockout	Impaired neuronal differentiation	Wu et al., 2010

NSC, neural stem cells, IIS, insulin and insulin-like signaling, DG, dentate gyrus, SVZ, sub ventricular zone, KO, knockout.

IGF-1 leads to increased brain size due to increased myelination (Carson et al., 1993). Finally, *Igf-1* null mice presented with decreased proliferation and differentiation of oligodendrocyte lineage (Ye et al., 2002).

Whether these effects persist into adulthood requires further investigation. Recently however, Chaker and colleagues showed that the inhibition of IGF-1 signaling in rodent adult olfactory bulb NSCs was able to hinder age-related stem cell decline and preserve the production and integration of newborn neurons (Chaker et al., 2015). IGF-1 was also shown to stimulate proliferation of adult hippocampal NSC both *in vivo* and *in vitro* while blocking the PI3K/Akt pathway stopped the proliferative effects of IGF-1 on NSCs (Åberg et al., 2000, 2003). This was later supported by Chigogora and colleagues finding a correlation between IGF-1 levels and an elevated risk of human depression (Chigogora et al., 2016), a disorder known to involve neurogenic and possible NSC deregulation (Hill et al., 2015). Furthermore, the deletion of the FoxO family members results in increased brain size and proliferation during development but also in a depletion of the progenitor pool and ultimately a decrease of SVZ adult neurogenesis (Paik et al., 2009; Renault et al., 2009). FoxO3 in particular seems to regulate quiescence of the adult SGL and SVZ NSC population and to have a role in oligodendrocyte regulation. FoxO transcription factors are also sensitive to oxygen changes making them ideal effectors between oxidative stress, a known aspect of aging, and stem cell maintenance (Renault et al., 2009). Besides these studies, several others have reported pro-neurogenic effects of insulin when investigating the mTOR pathway as discussed in Section mTOR.

Interestingly, IGF-II is produced by choroid plexus and released in the cerebrospinal fluid (CSF), allowing it to come in contact with the neurogenic niches. NSCs in the SVZ extend a process through the ventricular wall and come in contact with the CSF directly, thereby allowing its composition to directly alter their regulation. Increased presence of CSF IGF-II during development for example, promotes neurogenesis (Lehtinen et al., 2011; Ziegler et al., 2015). IGF-II is also involved in hippocampal neurogenesis in adulthood (Bracko et al., 2012). *In vitro* and *in vivo* studies showed IGF-II involvement in promoting NSC maintenance (Ziegler et al., 2012). Studies have also linked IGF-II dependent mechanisms to hippocampal-dependent memory retention (Chen et al., 2011) and more specifically to age-related cognitive decline (Steinmetz et al., 2016) in rodents, further supporting a link between IGF-II and NSCs function and presenting a key target for further researching seeking to preserve AHN during aging (See **Table 1**).

The overall effects of this pathway on NSC regulation however, remain inconclusive due to a limited number of concordant studies (Åberg et al., 2003; Itoh et al., 2012); some studies have reported increased IIS resulting in a beneficial increase in adult neurogenesis, for example during GH and IGF-1 mediated increases in neurogenesis as a result of exercise (Berg and Bang, 2004) or following a blueberry supplemented diet in rodents (Shukitt-Hale et al., 2015). In contrast, CR, which is known to directly target and diminish IIS, has also been proven beneficial for both cognition and longevity in rodent models of Alzheimer's disease (Parrella et al., 2013). **Figure 2** summarizes some of the NSC functions affected by the IIS pathway. As for mTOR, this highlights the need of a fine-tuned balance between IIS activation and inhibition throughout the lifespan. It is likely that several other factors such as oxidative state of the cell, biological age and brain region all play a role in this balance and can propel toward a positive or negative effect of the IIS pathway.

Sirtuins

Sirtuins are a group of deacetylases initially shown to extend lifespan in yeast by regulating mitochondrial function and cellular redox state (Aguilaniu et al., 2003). Deacetylases are key regulatory proteins as they can control the expression of several genes. Furthermore, sirtuin activity is NAD-dependent, making them likely candidates for the molecular link between metabolism and aging owing to their ability to respond to the cell's energy status. Indeed, Sir2 activation in yeasts mimics CR-induced longevity, which in turn was shown to depend on the Sirtuin pathway (Lin et al., 2000). Increased lifespan as a result of sirtuin overexpression was also confirmed in other organisms such as *Drosophila* and *C. elegans* (Guarente, 2007). Similarly, the mammalian components of the sirtuin family, SIRT1 and SIRT4 have also been implicated in CR diet-regulated processes, showing that the link between metabolism and aging could be conserved across species (Guarente, 2007). Research into Sirtuins also highlighted the importance of NAMPT, the rate limiting enzyme in mammalian NAD+ synthesis; aging is accompanied by chronic DNA damage which leads to NAD+ depletion, Sirt1 inactivation and thus mitochondrial dysfunction (Guarente, 2014). Overexpression of NAMPT can rescue NAD+ levels and counteract these changes as shown by interesting studies investigating its effects on the accelerated aging disorder, Cockanye syndrome (van der Veer et al., 2007; Guarente, 2014; Scheibye-Knudsen et al., 2014). The importance of NAD+ and sirtuins in aging was also supported by recent studies by Song and colleagues showing that NAMPT inhibition is sufficient to induce senescence in human fibroblasts (Song et al., 2015).

The above studies however did not focus on the effects of NAMPT on NSCs. Interestingly, a recent study showed that NAMPT ablation recapitulated aspects of NSC aging such as decreased NSC proliferation in rodents (Stein and Imai, 2014), suggesting that NAMPT and the sirtuin pathway play key roles in NSC aging as well. Studies are now beginning to integrate the role of sirtuins, aging and diet or nutrition on the CNS specifically. For example, increased Sirt1 levels in murine brains due to CR were shown to increase anxiety and decrease exploratory drive (Libert et al., 2011). Whilst NSC function was not assessed in these studies, these conditions are known to involve deregulation of hippocampal neurogenesis (Libert et al., 2011; Hill et al., 2015) making it a plausible underlying mechanism. These studies also highlighted a possible disadvantage of increased Sirt1 activity and CR. In line with this, when the effect of SIRT1 deletion was investigated in prion disease, a condition in which neurogenic deregulation is also implicated (Gomez-Nicola et al., 2014), it was found to delay disease onset and to prolong the healthy portion of the affected animals. This was mimicked by CR (Chen et al., 2008). In contrast, others have shown that sirtuins can have neuroprotective functions in response to neuronal damage and neurodegenerative conditions; firstly, SIRT1 is upregulated in mouse models of Alzheimer's disease and amyotrophic lateral sclerosis and shown to enhance neuronal survival both *in vitro* and *in vivo* (Kim et al., 2007). Furthermore, SIRT1 was also found responsible for neuroprotective effects in murine models of axonal injury (Araki et al., 2004). SIRT1 overexpression was found to replicate the beneficial effects of CR in the context of several neurodegenerative conditions in various animal models (Gräff et al., 2013).

As many neurodegenerative conditions experience changes in neurogenesis, it is likely Sirt1 conveys some of its effects by influencing NSC function. SIRT1 activation in particular was linked to changes in neurogenesis in the perinatal SVZ; oxidizing conditions were found to activate SIRT1 and push the progenitor pool toward astrocytic differentiation whereas a reducing environment would promote neuronal differentiation (Prozorovski et al., 2008). Similarly, blocking Sirt1 activity disengaged redox changes from SVZ NSC fate (Prozorovski et al., 2008). Rafalski et al. in contrast, showed that Sirt1 inactivation pushed NSCs toward an oligodendrocyte lineage (Rafalski et al., 2013). Following Prozorovski's results, it is possible that the neuroprotective effect of SIRT1 activation, in part, is explained by improved CNS support from an increased number of astrocytes (See **Table 1**). An interesting avenue would be to investigate whether this finding is replicated in the DG and how this SIRT1-mediated modulation of the NSC pool changes with age.

Finally, these findings are now being investigated in human populations, Libert and colleagues for example showed that rare human SIRT1 variants are associated with anxiety and mood disorders (Libert et al., 2011). Interestingly, Sirt1 is also one of the two genes recently implicated in major depressive disorder by whole-genome sequencing findings (Cai et al., 2015). Though dietary interventions such as CR seem to be the most potent effectors of Sirtuin activation, some nutrients, like polyphenols have also been identified to directly activate components of this family (Howitz et al., 2003). Together the studies reported above, suggest the relationship between Sirt1, CR, and aging is regulated by intricate mechanisms, which become even more complex when acting upon different types of NSCs. The key NSC's functions acted upon by the sirtuin pathway are reported in **Figure 2**.

EPIGENETICS

Epigenetics is at the forefront of aging research, a position supported by the recent establishment of an "epigenetic clock" by Steve Horvath, and has clear functions in adapting the organism's responses to its environment. These attributes make it a key

mechanism mediating cellular and molecular responses to diet and aging processes (Rea et al., 2016). Epigenetic modulation comprises many mechanisms and is usually identified as the driving force behind changes in gene expression, which are not due to DNA sequence mutations. Such a process clearly has very important functions in stem cell regulation of different tissues as it can confer lineage-determining decisions as well as maintain quiescence. Epigenetics also plays an important role in aging; twin studies showed that genetic background only has a 25% influence on longevity (Herskind et al., 1996) suggesting that the remaining effects would be dictated by the environment, which usually ensues its effects through epigenetics. A role for epigenetics is also supported by notion that the effect of genetic variations on cognition and brain structure increases with age, recently reviewed by Papenberg et al. (2015). One of such environmental factors able to cause epigenetic changes is likely to be diet.

A line of thought believes that as epigenetic changes are less permanent, it would be more efficient to target and reverse them rather than targeting any genetic mutations which may arise as a result of aging (Rando and Chang, 2012; Beerman and Rossi, 2015). By restoring a "young" epigenetic environment one may be able to reverse age-related deficit; in a similar manner to the aforementioned heterochronic parabiosis studies (Villeda et al., 2014). Another factor supporting the notion that targeting epigenetics may be an efficient way to reverse aging comes from studies showing that only a small number of loci are altered consistently throughout aging, allowing for targeted interventions rather than global ones (Beerman and Rossi, 2015). Furthermore, epigenetic changes could be used as aging biomarkers, providing important information on the aging rate of an organism, with the potential to enable more precise preventive strategies. It is therefore important for us to understand how age and nutrition affect epigenetics and how this causes alterations in NSC regulation, disease progression and longevity. For the remainder of this section we will evaluate the evidence for such a relationship.

Epigenetics and Nutrition

There are several studies showing perinatal or *in utero* nutrition can have vast effect on health later in life, mainly related to cardiovascular disease, diabetes and obesity (Choi and Friso, 2010). Interestingly, these effects were also witnessed in the offspring of the affected animals suggesting that epigenetic changes could be passed through generations. The involvement of epigenetics was also confirmed via methylome analysis, though a causal link is yet to be shown (Radford et al., 2014; Fontana and Partridge, 2015). Such mechanisms are set in place to ensure adaptation for the fetus to its environment—CR and DR prepares a fetus to food scarcity whilst over nutrition primes for a nutrient abundant environment. Studies have suggested it may be the mismatching of predicted and actual nutrient availability to cause some of the detrimental health effects later in life (Perera and Herbstman, 2011).

While epigenetic changes occurring during the malleable stages development in response to nutrition have been extensively studied, those happening during adulthood are less known. The evidence from developmental studies reported above, however shows that mechanisms are set in place for epigenetics to respond to dietary and nutritional cues through the lifespan. Indeed, diet can alter epigenetics in several ways, these include the donation of methyl groups and the regulation of several enzymes (Mathers and Ford, 2009). As well as the alterations in response to CR and DR, other dietary alterations can have effects on epigenetics, such as the intake of specific nutrients. Studies on agouti mice showed a methyl-supplemented diet was able to cause DNA hypermethylation, making it likely for diet-acquired methyl donors like choline and methionine (an essential amino acid) to have similar effects (Waterland and Jirtle, 2003; Niculescu et al., 2006; Waterland et al., 2007). Furthermore, the trace mineral zinc interacts with histone deacetylase regulation (HDAC) and causes their inhibition (Myzak et al., 2006) whilst resveratrol, a dietary phenol, activates the HDAC SIRT1 (Rafalski and Brunet, 2011). Vitamin D also appears to form an important link between nutrition, aging and epigenetics as its varying concentrations can delay the aging phenotype in mice and its mechanism of action is known to involve histone acetylases (HATs) and HDACs (Tuohimaa, 2009).

Epigenetics, Aging, and Neural Stem Cells

Several years ago it was found that cellular methyl content declines with increasing age in mammals (Wilson and Jones, 1983). This loss is likely to contribute to genomic instability, which is a hallmark of aging cells. Recently, Horvath and colleagues showed the existence of an epigenetic clock and developed a predictor able to estimate the methylation age of most tissues or cell types, suggesting that specific epigenetic changes occur as a result of age (Horvath, 2013).

Though there are a number of possible epigenetic marks, methylation is the mark considered to be the most stable one and thus the most likely candidate for encoding aging and nutritional changes. Methylation changes as a result of age can be divided into two main categories: those resulting from loss of fidelity when copying methylation marks and those arising from abnormal addition or removal of methylation marks. Further to this, a decrease in the activity and expression of the DNA methyltransferase DNMT1 was found with increasing age (Cooney, 1993). Interestingly, DNMT1 controls stem cell balance and lineage decisions in several tissues; its knock out in embryonic NSCs, for example, causes a preferential push toward astroglial differentiation (Fan et al., 2005). DNMT1 loss in general causes an aging phenotype highlighting it as a key molecule governing aging processes (Beerman and Rossi, 2015). In contrast, some housekeeping genes that are usually unmethylated seem to become methylated with age. This may be due to an increase in DNMT3 activity; DNMT3 is a methyltransferase responsible for de novo methylation, a process shown to be key in regulating stem cells as it halts self-renewal to allow differentiation. A DNMT3 knock out in mouse in fact showed impaired post-natal SVZ and SGL NSC differentiation (Wu et al., 2010) (See **Table 1**).

An important methylation mark, studied in the field of aging and NSCs, is the methylation of lysine 27 on histone 3 (H3K27).

The differing methylation of this mark in fact can regulate adult NSC differentiation; when this mark has a single methylation, transcription is enabled, when the mark has 2 or 3 methyl groups gene transcription is repressed (Zhang et al., 2014). Another epigenetic mark often involved in aging is histone acetylation; several histone acetylases (HATs) are key in regulating the NSC pool by affecting both proliferation and differentiation of the neural progenitors. Sirtuins as detailed above are part of this family. There is also evidence of different epigenetic marks influencing one another—i.e., DNA methylation being restricted by acetylation marks (Beerman and Rossi, 2015). See **Figure 2** for a representation of this interconnection. Mathers and Ford suggest that changes in methylation do not occur in every cell in a tissue. They explain how this leads to promoter methylation heterogeneity and thus to divergent gene expression and cellular response across different tissues with increasing age (Mathers and Ford, 2009). Together these studies show that epigenetic mechanisms are pivotal to a permissive or restricting environment for gene transcription in response to environmental cues and are therefore likely molecular effectors of nutrient intake and its ensuing effect on NSC regulation.

Finally, as well as sharing common downstream mechanisms, aging and nutrition can also affect one another; for example, nutrient intake may change as a result of age which could then cause the age-related epigenetic changes. A reduction of fruit and vegetable intake for instance, would reduce the intake of zinc and thus affect HDAC function (Mathers and Ford, 2009). Given the prominent role played by NSCs during aging, an exciting avenue would be to explore epigenetic changes in these cells in response to diet.

CONCLUSION

In conclusion, though much progress has been made in establishing the role played by nutrition in longevity, and on stem cells more broadly, its role in NSCs regulation is still to be elucidated. In this review we have discussed how mTOR/IIS pathway inhibition and sirtuin activation may enhance longevity and CNS function, an effect achieved at least in part, through their impact on NSC function. Importantly, we have described how these responses can be shaped by diet and nutrition.

Whilst the positive impacts of CR and IF continue to be detailed in model systems, more targeted pharmacological approaches may be beneficial for use in frail and elderly populations. This highlights the need for a more thorough understanding of the molecular pathways involved in these dietary paradigms.

Further to this, much more research into the genetic and epigenetic influences of diet and nutrition is required, to refine populations that potentially stand to gain the most from such interventions. Despite these caveats, there is much excitement in the field as dietary paradigms such as IF are employed in human studies and the ensuing encouraging results with regards to their impact on cognitive performance (Brandhorst et al., 2015; Fontana and Partridge, 2015).

AUTHOR CONTRIBUTIONS

The co-authors fulfill the criteria for authorship; they all contributed to the manuscript, approved it and agree to be accountable for its content. Cd, TM, and ST conceived the review, Cd carried out the literature review research, Cd and TM wrote the manuscript, ST revised the manuscript. The manuscript and parts of it, have not been, and will not be submitted elsewhere for publication.

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