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Editorial

Old and new tools to study human brain physiology: Current state, future directions and implications for metabolic regulation

In this issue of *Metabolism: Clinical and Experimental*, Nolde et al. [1] examines how the human brain responds differently to food cues when fasting or fed and how this, in turn, relates to changes in hormone levels. Activations of the orbitofrontal cortex to food cues with high ratings for craving vs. low ratings were associated with insulin and C-peptide levels when fasting and with glucose in the fed state, while activations of the caudate were associated with adiponectin in fasting [1]. This study uses fasting for 36 h to perturb the human energy homeostasis system and to alter a wide variety of hormones and then aims to determine how these relate to differences in the way the brain processes food cues and, by extension, how it controls energy and metabolic balance.

In this study, fasting was used to perturb the system [1] and thus to determine how the way the brain responds to food and food cravings is altered. Fasting may alter activations of the hypothalamus, as part of the homeostatic system, through changes in hormones. While this study examined hormones related to glucose control, including insulin, glucose, c-peptide, and adiponectin, many other hormones, including the prototypical adipose tissue secreted hormone leptin, are altered by fasting and would, in turn, alter the way the brain responds to food cues.

A major effector of food intake in rodents- with a significant but more limited role in humans- is leptin. Leptin is secreted by adipose tissue, circulates at levels proportional to the amount of fat mass and is decreased very quickly with acute decreases in caloric intake, i.e. fasting [2–5]. In two days, leptin levels would have dropped by about 50% and potentially increased the drive to eat [5]. Leptin is known to alter brain activations to food cues when administered (e.g. a mechanism for perturbing the system) to lean, hypoleptinemic women in not only the hypothalamus but also in other brain areas related to attention and reward [6]. Ghrelin, amylin, irisin, and glucagon-like peptide 1 (GLP-1) are other molecules that, in addition to the glucose-related molecules studied in the most recent article, are impacted by fasting and/or obesity and may also have effects in the brain [7–28]. To directly study the effects of each specific molecule, one would need to either administer, ideally in a placebo-controlled manner, the specific molecule, its antagonist, or a third medication that is known to alter in a specific manner the circulating levels and/or target actions of the molecule of interest.

Another factor that needs to be considered in human studies, as compared to those in rodents, is that ethical considerations limit the extent to which the human brain can be studied (e.g. it is not possible to place electrodes or to dissect healthy human brains). Functional magnetic resonance imaging (fMRI) is one of the most widely used tools we currently have to study eating behaviors/appetite in the human brain. Combined with mechanisms to perturb the human system, this tool can yield interesting data. While one "tool" for perturbing the system is altering food intake (e.g. fasting vs. fed) and, thus, many hormones and other related outcomes, additional "tools" that can be used in a smart way to elucidate more fine-tuned mechanisms come in the form of hormones or medications that may alter the activity of a specific hormone or its receptor. By giving a medication that acts on a particular hormone and combining this with non-invasive techniques such as fMRI, researchers can now gain more information on how that specific hormone acts in the human brain.

fMRI has high spatial resolution and can be combined with tasks to provoke specific brain activations- such as the food images utilized in this study- but it has less clear temporal resolution and is not a direct measure of brain activity. When neurons are active, they use oxygen from the blood, causing an increased flow of oxygenated blood to a recently active brain area. This principle, and changes in magnetization resulting from shifts between oxygenated and deoxygenated blood, underlies the use of fMRI as an indirect measure of neural activity. Beyond these, artifacts from the throat, sinuses, and eyes make detecting activations in the hypothalamus [29,36] and orbitofrontal cortex [30] more difficult. Although, clearly, more research is needed in this area, recent studies have also seen poor to moderate test-retest reproducibility with fMRI and food cues in overweight and obese men and women [31], with similar findings observed in BOLD signals to reward-related activations [32], suggesting a need for larger sample sizes, well-balanced study designs and a preference for cross over study designs, which allow within subject comparisons, where feasible. In addition to the current study showing changes in activation with fasting, the time of day may also matter in terms of brain activations, as demonstrated by another study in women [33] and thus time of the study needs to be standardized. However, while analytical methods and more advanced scanners and head coils have been designed to overcome the artifacts around the hypothalamus and orbitofrontal cortex, overcoming

Abbreviations: ACTH, adrenocorticotropic hormone; AgRP, agouti-related peptide; ARC, arcuate nucleus; CART, cocaine- and amphetamine-related transcript; CNS, central nervous system; EEG, electroencephalogram; FDA, Food and Drug Administration; fMRI, functional magnetic resonance imaging; GnRH, gonadotropin releasing hormone; GH, growth hormone; GLP-1, glucagon-like peptide 1; HSDD, Hypoactive Sexual Desire Disorder; IGF, insulin-like growth factor; IGF-BP, IGF binding protein; LH, leutinizing hormone; MC, melanocortins; MCR, melanocortin receptors; MEG, magnetoencephalogram; MSH, melanocyte-stimulating hormones; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; PCSK, proprotein convertase subtilisin/kexin; PET, positron emission tomography; SPECT, single photon emission computed tomography; T3, trioodothryonine; T4, thyroxine; TSH, thyroid-stimulating hormone; US, United States

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the limitations of temporal resolution and from the indirect nature of fMRI signals is more difficult.

Another way to address these limitations is to use or combine other methods with fMRI. For instance, magnetoencephalogram (MEG) and electroencephalogram (EEG) non-invasively detect brain activation by the magnetic fields around electrical signals or electoral signals directly, respectively, with great temporal but not spatial resolution and can be combined with fMRI to get the best of both worlds [34,35]. MEG and EEG are limited, however, in terms of the depth with which activity can be detected in the brain, making areas such as the hypothalamus difficult to study. There is also the more invasive positron emission tomography (PET) and single photon emission computed tomography (SPECT) techniques, which use radioactive tracers to examine where the binding potentials of specific molecules, such as neurotransmitters or receptors, are located in the brain without temporal resolution but with acceptable spatial resolution. Beyond these mechanisms, the least invasive and easiest methods to conduct are neurocognitive testing, i.e. computer tasks designed to test a network of activity with directly measuring the brain, which is often used in combination with the above techniques.

While all of these methods have been utilized to study the brain in obesity as it applies to appetitive behaviors, combinations of these may be best to dissect molecular pathways regulating energy homeostasis in humans in order to better define how molecules and potential therapeutics act in human brains. Although there may be limitations in directly studying the hypothalamus in humans with fMRI due to its small size and location next to the sinuses, which can create artifacts [29,36], our group and others have observed changes in some studies with fMRI focusing on regions of interest (ROI) that provide validation in humans of prior findings in mice [6,36,37]. These studies also provide evidence that one can utilize hormones and medications which target molecules known to act in the hypothalamus and observe how they alter brain processing using the techniques above to determine whether the hypothalamus- and/or other, higher order brain areas- are activated/altered in humans. Importantly, beyond any peripherally secreted hormones that may be administered to humans, administration of specific neuropeptides that cross the blood brain barrier into the hypothalamus can also be utilized to study hypothalamic/homeostatic activity in the human brain and elucidate molecular pathways in humans e.g. neuropeptides acting on receptors downstream of leptin and mediating leptin's effects in human brains.

In a non-leptin-tolerant system, leptin primarily acts, on the basis of experiments in rodents, in the arcuate (ARC) nucleus of the hypothalamus to inhibit neurons which release agouti-related peptide (AgRP) and neuropeptide Y (NPY) and to increase the activity of neurons that release pro-opiomelanocortin (POMC) and cocaine- and amphetamine-related transcript (CART), to decrease energy intake [38–40] (Fig. 1B). POMC is cleaved by proprotein convertase subtilisin/kexin 1 (PCSK1) to produce adrenocorticotropic hormone (ACTH) and γ-melanocyte-stimulating hormones (γ-MSH) and then further by PCSK2 to produce α-MSH and β-MSH [41] (Fig. 1A). ACTH and the MSHs (α, β, and γ) make up the "melanocortins" (MC) [41]. POMC is downstream of leptin, a hormone primarily secreted by adipocytes whose circulating levels reflect primarily the amount of body fat stored in the body and secondarily acute changes in energy intake [42]. Although it is currently presumed that neuropeptide changes in rodents and other animal species correspond to identical changes in the brains of humans this, strictly speaking, has not yet been proven beyond any doubt given lack of appropriate studies in humans. Aside from energy intake/expenditure, MCs, located downstream of leptin in the system conveying leptin's actions, have been hypothesized to play several other roles, which remain to be fully defined in humans. Importantly, leptin is known to influence not only energy intake/appetite and energy expenditure but also the hypothalamic-pituitary-peripheral axes relating to gonadal function, growth hormone, thyroid, and adrenal systems in animals, although its role in humans is apparently more limited in terms of pathways activated [43–46]. Notably, leptin acts through both the POMC/MC system and the AgRP/NPY system, and so which effects go through the MC system vs. the AgRP/NPY system needs to be determined.

Molecules which may mimic AgRP/NPY or their antagonists can be developed for clinical use in human physiology studies, and the MC system, which is currently being targeted by pharmaceutical companies for the development of agents as medications lends itself even more for physiology studies in humans. Bremelanotide (PT-141, Vylessi™) was recently approved by the United States (US) Food and Drug Administration (FDA) for Hypoactive Sexual Desire Disorder (HSDD; [47]) in women. This newly approved medication is the second melanocortin (MC) related medication and the first in the central nervous system (CNS) targeting class of MC receptor (MCR) agonists and, as such, this recent approval has raised many questions as to the role of the MC system in the CNS in human reproduction, metabolism, with respect to the molecular pathways activated in the brain to mediate its actions, as well as related safety and efficacy.

In more detail, POMC is cleaved to create each of the MCs in a broadly tissue-specific manner, where some tissues will produce all or some of the MCs [48]. In the hypothalamus, POMC is cleaved into all of the MCs, α-, β-, and γ-MSH as well as ACTH [49–51], where they act downstream of leptin to increase energy expenditure and decrease energy intake in animal studies and to possibly stimulate/alter certain hypothalamic-pituitary-peripheral axes [41]. MCs act on the melanocortin receptors 1–5 (MC1-5R), which are located throughout the body. In the simplest terms, MC1R are located on melanocytes, MC2R on the adrenal cortex, MC3R in the brain, gastrointestinal tract, and kidney, MC4R in the brain, and MC5R on exocrine cells [52,53], although tissue expression in rodents and humans have shown more widespread locations with different levels of expression in different tissues [49]. In terms of binding, α-MSH has a higher affinity than γ-MSH, ACTH, or β-MSH for MC1R and MC5R though MC5R has an overall lower affinity than MC1R, ACTH is the only MC with affinity for MC2R, γ-MSH, ACTH, and β-MSH has a higher affinity than α-MSH for MC3R, and β-MSH has a higher affinity than α-MSH, which has a higher affinity than ACTH which has a higher affinity than γ-MSH for the MC4R [54,55]. However, the key receptors being targeted by MCR agonists, including the newly approved medication and others in the clinical trials stage, are MC3R and MC4R, which are primarily located in the brain.

Bremelanotide, the most recently approved MC-targeting medication, binds relatively strongly to MC1R, MC3R, and MC4R, but may also bind to the other MCRs with less affinity [53,56]. The actions of bremelanotide on sexual function (e.g. erections, HSDD) have been attributed to actions on MC3R and MC4R from studies in rodents [56]. However, exact mechanisms and other outcomes of bremelanotide binding nonspecifically remain to be determined.

Two medications which target MCRs are also approved and/or in development beyond Phase I. Others have been discontinued from the pipeline and/or are in preclinical or Phase I stages. Afamelanotide (melanotan I, CUV1647, Scenesse®) is an approved α-MSH analogue that acts primarily on MC1R but also MC3R and MC4R, currently used for the treatment of Erythropoietic Protoporphyria [57–59]. Setmelanotide (RM-493), an MC4R agonist with more specific activity in comparison to the less specific binding of bremelanotide, has been shown to be effective in individuals with POMC and/or leptin receptor deficiency in terms of body weight regulation (similar to past studies of leptin deficiency) [60–64]. In a small, 72-hour study ($n = 12$) with patients who were obese, RM-493 did alter energy homeostasis [65], and needs to be studied further in terms of both energy intake/expenditure in larger and longer-term studies. Questions as to the role and downstream hormonal and metabolic effects of agonism for specific MCRs, i.e. the specific binding of setmelanotide for MC4R vs. the general binding of afamelanotide and bremelanotide, remain to be answered. Similarly, the role for these medications in weight loss for non-syndromic obesity in humans remains to be clarified.

Many questions remain about the effects and usage of MCR agonists in humans. Future research will need to determine their impacts on energy

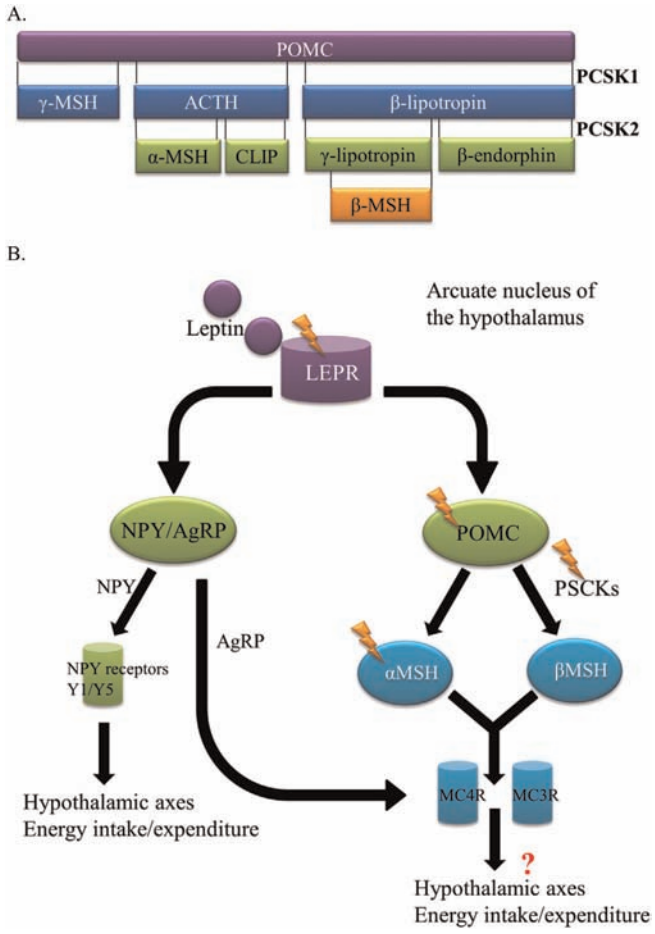

Fig. 1. A. POMC is cleaved by PCSKs to produce ACTH and the MSHs which are considered the melanocortins and act on the melanocortin receptors. B. Leptin exerts its actions through the leptin receptor and POMC or AgRP/NPY pathways to impact the hypothalamic-pituitary-peripheral pathways and regulate energy intake/expenditure. Mutations in typical obesity have been found along the pathway (orange lightning). New medications are targeting the melanocortin receptors (MC4R/MC3R) in the brain, and thus, it is necessary to determine which of leptin's effects go through this pathway (question mark).

intake/energy expenditure in both leptin sensitive and leptin tolerant (e.g. obesity) systems as well as any effects on the hypothalamic-pituitary-peripheral axes. Mechanisms for bremelanotide, and other MCR agonists, will need to be further defined and delineated in humans, as acknowledged by the FDA report, and many physicians still have questions about the level of efficacy (which was modest in the trials) that remains to be further studied in the future. Finally, these compounds can be used as useful tools to dissect the specific physiological role of the MC vs. the AgRP/NPY systems in mediating the effects of leptin in the human brain, which may or may not be with the same as their role in rodents.

1. Body weight

Notably, leptin tolerance, indicating a plateauing effect in the body weight regulation by leptin, where leptin's effects increase as leptin levels increase from the hypoleptinemic to normoleptinemic range but reach a plateau and thus cannot induce further weight loss when administered in doses that result in even higher levels, has prevented the use of leptin analogues or agonists in humans with obesity [66–68]. Many potential mechanisms for leptin tolerance or resistance have been proposed [69–71]. It is generally observed that most people with obesity have high leptin levels [72,73] reflecting leptin tolerance and/or its ineffectiveness in obesity. The question remains as to whether MCs will be efficacious in these same individuals, which would be the case if the factor blocking leptin's effects is upstream of the MC receptors, or whether their administration would have limitations similar to those encountered in response to leptin administration.

Data linking MC agonists with energy expenditure and intake is accumulating. Many studies have shown that leptin administration in rodents increases energy expenditure and decreases energy intake [74–76]. Our group has documented that metreleptin decreases appetite and energy intake in response to normalization of leptin levels in hypoleptinemic women who have very low leptin [6] and that metreleptin does not change resting metabolic rate and/or activate the sympathetic nervous system [77]. In rodent studies, MCR agonists have been found to increase energy expenditure and decrease caloric intake [78–80]. In fact, our group was one of the first to examine how a MCR agonist, melanotetan II (which was later discontinued from development), acted in lean and obese mice to decrease food intake and increase energy expenditure, as well as improve insulin sensitivity by acting primarily in the CNS [79,80]. These findings have been extended in a small sample of obese, but otherwise healthy, individuals without any known mutations who were given a melanocortin receptor agonist, setmelanotide, and found to have increased resting energy expenditure and decreased energy intake, resulting in weight loss [65]. These observations need to be expanded in larger human studies and extended to determine which of the other impacts of leptin are enacted by MCR agonists in first lean hypoleptinemic and later normoleptinemic and hyperleptinemic (obese) humans.

2. Reproductive hormones and sexual function

Little is yet known about how MCR agonists may impact the hypothalamic-pituitary-peripheral axes which are altered by leptin in hypoleptinemic humans. Most significantly, in relation to the indication for bremelanotide would be any impacts on the hypothalamic-pituitary-gonadal axis. States of induced leptin deficiency such as fasting during dieting, or more chronically in states of negative energy intake such as in hypothalamic amenorrhea and anorexia nervosa, decreases the frequency of secretory pulses of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) [81–83]. Decreased serum LH in turn is accompanied by decreased serum gonadal hormones to approximately 50% of baseline [84]. Short-term dieting can cause a reduction in gonadotropin levels by approximately 30% [85]. In rodents, our group has shown that leptin replacement during fasting prevents the fall of the gonadal hormones [86]. We have also confirmed this same restoration in humans through a series of experiments [5,43–46,87]. There is also evidence from rodents that MC3/4R knock-out models show dysregulated gonadal hormones [88–93], but whether MCs fully mediate the regulatory role of leptin in the hypothalamic-pituitary-gonadal axis would need to be confirmed through systematic well designed physiology studies in humans with congenital or acquired hypoleptinemia. Although whether MCR agonists may alter these hormones in states of energy deficiency and hypoleptinemia only vs. states of energy sufficiency or excess remains unknown; considering their effects on sexual function, this needs to be studied. It also needs to be studied whether their efficacy is higher in energy deficiency states that are also by definition hypoleptinemic states vs. whether their effect plateaus after a certain body weight that would correspond to when euleptinemia is reached.

3. Other hormones

The hypothalamic-pituitary-thyroid axis is impacted by low leptin states [43–46]. Our group has shown that leptin increases triiodothyronine (T3) without changing thyroid-stimulating hormone (TSH) or thyroxine (T4) first in rodents and then in lean men and leptin deficient women [5,43–46,86] but did not see this effect in leptin-replete humans [87]. Rodents given a MC4R antagonist did not show changes in TSH but only a small, transient change in T4 which recovered quickly [94].

Rodent knock-outs for the MCR do show mild effects on thyroid hormones and there is some evidence in rodent fasting studies that AGRP, NPY, and αMSH may impact thyroid hormones [94,95]. These effects need to be studied in humans, and these studies are only now possible given the availability of centrally acting MCR agonists.

The hypothalamic axis for growth is known to be inhibited during prolonged fasting in children and adolescents. Although fasting increases total and pulsatile growth hormone (GH) production, pulse frequency, pulse amplitude, and interpulse levels [96], circulating concentrations of insulin-like growth factor 1 (IGF-1), a hormone that stimulates amino acid uptake and protein synthesis while inhibiting lipolysis, are decreased [97], indicating an end organ resistance to the effects of GH [98]. However, in longer-term studies of leptin deficient individuals, leptin replacement did increase IGF-1 and IGF binding protein (IGF-BP) levels, more specifically, IGFBP-3 [99]. Less is known about whether and, if yes, how MCs may impact the growth hormone (GH)-IGF system vs. whether other pathways downstream of leptin (e.g. NPY or other) may mediate leptin's effects. MC4R mutations in rodents suppressed the GH-IGF axis [100]. MC4R mutations in humans are associated with differences in height and minor differences in GH levels, but no changes were seen in IGF-1, IGF-2, or IGFBP3 [101]. All other IGFBPs, intact or total, have not been studied to date in humans with mutations. Future studies would need to determine the effects of MCR agonists on this axis in humans with leptin deficiency and by extension MC deficiency where any effects of replacement with MC agonists would be expected to be maximal and/or in subjects without these mutations who may be administered such agonists as part of physiology studies and/or in real-world trials in subjects who are taking this as a treatment for HSDD.

In terms of the hypothalamic-pituitary-adrenal axis, we have shown that leptin replacement decreases corticosterone and ACTH in rodents [86]; we found that leptin did not have the same effect in normoleptinemic humans in the short-term [5,87], although it did decrease cortisol in hypoleptinemic women in the long-term [43]. Less is known about impacts of MCR agonists on this axis. However, patients who have mutations in the POMC gene have hypocortisolism [64], which may suggest that it could have actions in those cases, potentially secondary to other changes. This axis will need to be studied further to define the role of MCR agonists in humans, but considering the lack of effects of leptin, it would be unexpected to impact ACTH or cortisol.

4. Questions on safety and efficacy

In women, for which approval was given, bremelanotide as compared to placebo showed a 0.5 placebo-subtracted increase in satisfying sexual events per month as well as 1.7 placebo-subtracted increase in the female sexual function index and a 4.3 placebo-subtracted decrease in female sexual distress with relatively minor adverse events, such as headache, nausea, flushing and mild injection-site reactions [102]. An earlier, smaller pilot in women has similar findings with increases in reported arousal and sexual desire with bremelanotide [103]. In studies in men, intranasal PT-141 (setmelanotide) alone or with sildenafil increased erectile response with similar side effects, such as flushing, nausea, and headache [104–106]. Similarly, intranasal bremelanotide was found to be generally safe and tolerable in a 2017 Phase I trial in men and women with similar mild reactions as above in flushing, headaches, etc. [107]. Side effects from afamelanotide were mild and considered tolerable, such as headache, nausea, and nasopharyngitis [57,59]. Overall, MCR agonists appear to be relatively safe with good efficacy. However, potential other effects need to be studied, such as actions in brain and on hypothalamic-pituitary-peripheral axes, as well as differences with general vs. specific activations of MCRs. It also remains to be determined whether the effects of these medications are mediated through changes in sex hormone levels and/or other peripheral hormonal changes.

5. Summary

In conclusion, perturbations of the human system are necessary to understand how appetitive and eating behaviors are implement in the CNS and translated into obesity. The present article published in Metabolism explores impacts of large-scale changes in the shift of broad hormonal systems that happens with fasting, pointing out associations with glucose metabolism related molecules [1]. Following this article, targeting the system with specific hormones and neuropeptides would be necessary to fully understand how these brain activation shifts occur with fasting.

While MCR agonists are beginning to receive approvals by the FDA, with one previously approved, one more recently approved, and others that may receive future approvals for other indications (e.g. setmelanotide for obesity), it is absolutely necessary to study not only safety and efficacy of these medications which is the focus of FDA mandated and industry run clinical trials, but also to study in depth mechanisms and molecular and hormonal pathways activated to understand how these systems work in the human system. As a downstream effector of leptin, MCs likely have many effects beyond those currently being studied, though these remain to be defined. One could also speculate that similar to leptin the effect of MCs could also be permissive, and may plateau at or above a given body weight to the extent that, for a given physiological function, they reflect and mediate leptin's effects. These and other related questions will certainly make human translational research on MCs fruitful and exciting in the years to come.

Given the current techniques, there are many ways to confirm, extend, and/or refute findings in rodents with appropriately designed studies in humans. While fMRI remains one of the best current methods for observing brain activity in humans, alone or in combination with other measures such as EEG or MEG, individual variability has made test-retest reliability of fMRI difficult and thus calls for the need of larger subjects, cross-over designs, standardized timing and eating conditions, and eventually better methods to measure brain activity. Although each technique to measure brain activity in humans has its own limitations, improvements in each method, along with the most coordinated use of combinations of available methods and intensive research towards the development of novel methods, can propel clinical research forward in the coming years.

Future research should also involve perturbations of the system with medications, hormones, and/or neuropeptides along with combinations of neurocognitive and neuroimaging techniques to provide a more comprehensive view of human brain physiology with a clear aim towards finding effective solutions for unmet clinical needs such as obesity and its metabolic complications that include neurocognitive diseases such as Alzheimer's disease.

Disclosure statement

The authors have nothing to disclose.author153957]

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