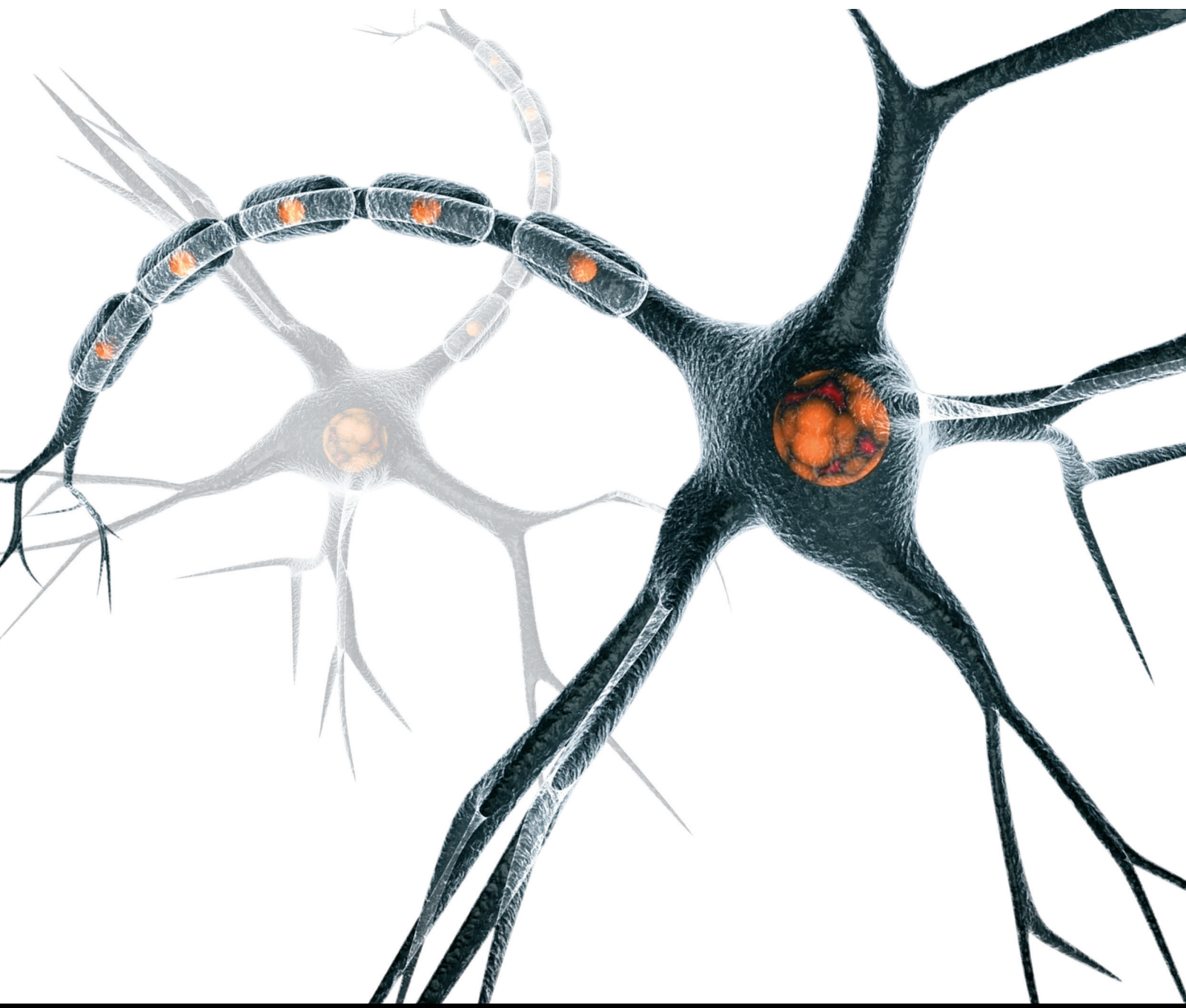
Neural Plasticity

Neural Plasticity in Obesity and Psychiatric Disorders

Guest Editors: Mauricio Arcos-Burgos, Maria T. Acosta, Ariel F. Martinez, Maximilian Muenke, Pablo J. Enriori, and Claudio A. Mastronardi



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Review Article

Obesity Reduces Cognitive and Motor Functions across the Lifespan

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Due to a sedentary lifestyle, more and more people are becoming obese nowadays. In addition to health-related problems, obesity can also impair cognition and motor performance. Previous results have shown that obesity mainly affects cognition and motor behaviors through altering brain functions and musculoskeletal system, respectively. Many factors, such as insulin/leptin dysregulation and inflammation, mediate the effect of obesity and cognition and motor behaviors. Substantial evidence has suggested exercise to be an effective way to improve obesity and related cognitive and motor dysfunctions. This paper aims to discuss the association of obesity with cognition and motor behaviors and its underlying mechanisms. Following this, mechanisms of exercise to improve obesity-related dysfunctions are described. Finally, implications and future research direction are raised.

1. Introduction

Obesity is the overaccumulation of fat which has aversive effects on health. The World Health Organization (WHO) defines overweight and obesity as body mass index (BMI) ≥ 25 and BMI ≥ 30, respectively [1]. Around the world, obesity has become a worrying health and social issue, threatening lives of thousands of people. According to the WHO [1], over 1.9 billion adults (39% adults) were overweight among which more than 600 million (13% adults) were obese. Childhood obesity is also common that 42 million children were overweight or obese in 2013 [1]. Considering its high prevalence, it is pressing to study the pathogenesis, manifestations, and prevention of obesity.

Obesity is related to a range of health-related problems, such as diabetes, heart disease, hypertension, and cancer [2]. Compared to normal-weight individuals, obese individuals have a reduced life expectancy [3]. Obese children show greater cardiovascular risk factors and persistence of obesity into their adulthood, which may be associated with higher likelihood of premature mortality [4, 5]. In addition to health problems, obesity is associated with poorer cognition and motor control, and altered brain plasticity. In this review, we first look into the behavioral manifestations of obese individuals' cognition and motor control capabilities. Next, obesity-related changes in brain plasticity will be discussed. Following this, the effects of physical exercise to combat obesity and obesity-related deficits in cognition and motor control will also be described. Finally, implications and future research directions are raised.

2. Cognition

Overweight and obesity are usually related to poorer cognition across lifespan [6–8]; however, the association between BMI and cognitive function is weaker in old age [9, 10], partly due to inaccurate adiposity measurement in the aged people [11]. Indirect evidence has shown an association between western high fat diet and impaired cognitive functions [12]. Based on BMI data, individuals who are overweight or obese

fall in the lowest quartile of global cognition, verbal fluency, delayed recall, immediate logical memory, and intelligence [13].

Other than BMI, other adiposity measures are also related to cognitive performance and brain changes. Visceral adiposity is inversely correlated with verbal memory and attention. High visceral adiposity is associated with smaller hippocampus and larger ventricular volume [14]. There is also a negative correlation between waist-to-hip ratio and hippocampal volume and a positive correlation between waist-to-hip ratio and white matter hyperintensities [15]. Compared to BMI, central adiposity has a stronger association with the risk of developing cognitive impairment and dementia in women [16]. Hence, studies using BMI as the only indicator of obesity may not be sensitive enough to capture obesity-induced cognitive dysfunctions.

Neuroimaging studies demonstrate atrophy in the frontal lobes, anterior cingulate gyrus, hippocampus, and thalamus in older obese individuals [17]. BMI increase is associated with lower metabolic activity in the prefrontal cortex and cingulate gyrus, smaller gray matter volume in many brain regions (particularly prefrontal cortex), and deficient white matter integrity in the uncinated fasciculus which is a structure connecting the frontal and temporal lobes [18–22]. Smaller gray matter volume in the left orbitofrontal region is related to poorer executive performance in obese women [21].

Childhood obesity is related to the reduced executive function, attention, mental rotation, mathematics, and reading achievement [23–25]. Obese adolescents have deficits in a range of cognitive functions, such as attention and executive functions [26, 27]. An animal study shows that high fat diet induces similar morphometric and metabolic changes in juvenile and adult mice; however, only early exposure to high fat diet hurts relational memory flexibility and decreases neurogenesis [28]. Thus, early exposure to high fat diet may be particularly deleterious to cognition.

People with higher midlife BMI have lower global cognition than their thinner counterparts [29] and midlife obesity is related to the accelerated cognitive aging, but this association is weaker in older adulthood [30]. Both age and BMI contribute independently to decreased brain volume in middle and older adulthood [31]. It is more likely for an older adult to have lower cognitive abilities if he/she was overweight or obese during middle age [32, 33]. Midlife obesity is related to an increased pace of deterioration in executive functions and an increase in waist-to-hip ratio is associated with substantial reduction in total brain volume [34]. Lower BMI and waist circumference and higher fat-free mass are associated with slower cognitive decline [35]. Midlife overweight/obesity, particularly with metabolic abnormality, is associated with higher dementia risk in older adulthood [33, 36–40]. Moreover, high midlife BMI is related to neuron and myelin abnormalities [41]. Hence, midlife is a critical period in which the overweight/obese status can predict one's cognitive functions and brain health in later life [42].

3. Motor Control

Besides cognition, obesity also affects motor control capabilities, degrading daily functions and health [43]. Children who are obese or overweight are poorer in gross and fine motor control and have delayed motor development [44–50]. Obese boys have poorer motor skills and a reduced activity of daily living [51]. Obese girls of 6th and 7th grades participate in less physical activity and have lower enjoyment of physical activity [52]. Children with high BMI have lower level of run which is a fundamental motor skill based on which complex motor skills are learned [53]. Cliff et al. [54] observe that the prevalence of mastery of all fundamental motor skills is lower in overweight/obese children, especially for run, slide, hop, dribble, and kick. In addition to BMI, waist circumference is also related to children's and adolescents' ability to perform fundamental motor skills [55]. There is an inverse relationship of BMI with fine motor precision, balance, running speed and agility, and strength in the 1st graders [56]. Obese children also have difficulty in postural coordination and a heightened dependency on vision during locomotion which is rather automatic in nonobese children [57, 58].

Adiposity is related to muscle quality ratio that is associated with motor conduction velocity and finger tapping speed [59]. Obesity is related to greater fluctuation in handgrip force production [60]. Subcutaneous fatness can account for a significant variance of health-related and motor fitness [61]. Excessive fat mass is associated with poorer posture and walking [62]. In middle and older adults, a combination of high BMI (or waist circumference) and high blood pressure is related to lower motor speed and manual dexterity [63]. During postural control, obese individuals require greater attention resources to maintain balance during unipedal stance [64]; this implicates that obese people consume attention resources to compensate for their motor deficits.

4. Obesity-Related Changes in Brain Plasticity

A number of factors may mediate obesity's effects on cognition and motor behaviors. For example, obesity may affect brain structure, leptin and insulin dysregulation, oxidative stress, cerebrovascular function, blood-brain barrier, and inflammation [11, 65–71]. Some also suggest that obesity-related changes in metabolism interact with age to impair brain functions [72].

In terms of brain structure, obese individuals have lower cortical thickness in the left superior frontal and right medial orbitofrontal cortex. The volumes of ventral diencephalon and brainstem are also reduced in obese people [73]. There is also a negative relationship between neuronal injury and gray matter density in hippocampus and cerebellum in overweight and obese individuals [74]. It is suggested that the medial orbitofrontal cortex, hippocampus, and cerebellum are involved in reward-based learning, memory, and motor control and learning [75–77]; structural alterations in these regions may be associated with deficits in cognitive and motor domains. Hitherto, the mechanisms underlying obesity's effects on brain structure are not clear.

High fat diet increases oxidative stress and inflammatory signaling in the brain [78]. Diet-induced obesity promotes reactive oxygen species in the brain which is associated with both body weight and adiposity [79, 80]. In children, intake of saturated fatty acids impairs both relational and item memory [81]. Occurrence of 15-week obesity during childhood can induce permanent epigenetic changes in rat's brain [82]. In rats, triglycerides diminish the passage of insulin-like growth factors (IGFs) into the brain through cerebrospinal fluid, impair hippocampal long-term potentiation, and impede leptin transportation across the blood-brain barrier [83–85]. Juvenile exposure to high fat diet impairs long-term spatial memory, but not short-term memory, suggesting a selective impairment of consolidation which is likely contributed by increased proinflammatory cytokine expression in the hippocampus [86]. Moreover, consumption of western diet is thought to degrade blood-brain barrier, which consequently damages hippocampus and leads to dementia [87]. Relative to those having normal diet, mice consuming high fat diet for 17 days develop insulin resistance in cerebral cortex tissues, degraded synaptic integrity, and poorer spatial memory [88].

Leptin is a cytokine and satiety hormone helping regulate appetite and energy expenditure. It can cross the blood-brain barrier and binds to presynaptic GABAergic neurons to produce its effects [89, 90]. Leptin production is increased in obesity [91]. As leptin receptors are widespread in the brain (e.g., throughout the cortex and the hippocampus), leptin can modulate memory processes [92]. Obese mice with impaired leptin signaling have deficits of hippocampal-dependent memory [93] and increased basal hippocampal inflammation [94, 95]. Leptin is related to neurogenesis, axonal growth, and synaptogenesis in addition to hypothalamic functions [96–98]. For hippocampal neurons, leptin plays a role in long-term potentiation and depression and thus is important for synaptic plasticity [92, 99, 100]. Compared to those with low leptin level, the elderly with high leptin level show less cognitive decline during aging [101]. High leptin level in individuals with small waist circumference is related to less cognitive decline over 10 years [102]. The presence of leptin may decrease the production of amyloid and speed up the removal of β-amyloid [103]. Older adults with higher leptin level are at a lower risk of developing dementia [104]. Obese individuals usually develop leptin resistance [105] which results in an increase in food intake and alteration of energy expenditure [90].

The circulating levels of insulin and signaling pathway are altered in obesity; this interacts with inflammatory processes to modulate cognition and behaviors [106]. Insulin plays a role in modulating hippocampal synaptic plasticity [107]. As insulin receptors are widespread in hippocampal and cortical brain structures, insulin signaling can contribute to the formation of declarative memory [108]. Insulin concentrations vary with adiposity and there is a negative relationship between the amount of visceral fat and insulin sensitivity [109]. Insulin resistance can result from high fat consumption or obesity [110, 111]. Dysfunctional insulin signaling can induce inflammation and promote β-amyloid and tau pathology, contributing to neurodegeneration [112, 113]. Insulin resistance can mediate cognitive impairment and neurodegeneration as insulin and IGFs can regulate neuronal survival, metabolism, and brain plasticity [114, 115]. During insulin resistance, there is a failure of cells to metabolize glucose, which consequently triggers an increase of insulin level. Insulin signaling is related to tau phosphorylation, an early pathology of Alzheimer's disease [116, 117]; this is complementary to the fact that there are a large number of insulin-sensitive glucose transporters in the medial temporal lobe [118]. Thus, insulin dysregulation in the obese people likely confers a greater risk of dementia to them.

The adipose tissue produces many substances for metabolism (adipokines, such as BDNF) and inflammation (cytokines, such as leptin). Many cytokines, such as interleukin-1, produced by the adipose tissue can cross the blood-brain barrier and affect cognitive functions through neuroinflammation [95, 119]. Adiponectin is involved in regulating glucose level and fatty acid breakdown. Similar to leptin, it exerts its effects in the brain to bring about weight reduction [120]. Its level is negatively associated with adiposity and can protect hippocampal cells [119]. Reduced hippocampal adiponectin levels are observed in aging animals, independent of high fat diet intake [121]. Thus, adiponectin is important for neurodegeneration prevention.

Neurotrophins, such as IGF-1 and BDNF, can mediate obesity's effects on cognition and behaviors. IGF-1 is mainly produced in liver and binds to the IGF-1 or insulin receptors to exert its effects to stimulate cell growth and proliferation and promote β-amyloid clearance in the brain [122]. Obese individuals usually show IGF-1 resistance, degrading their capability to prevent β-amyloid deposition and neurodegeneration [114, 123]. Besides, BDNF can bind to many receptors, such as TrkB and LNGF receptors, to support neuronal survival and stimulate neurogenesis and synaptogenesis [124–126]. Cardiometabolic diseases are usually associated with low BDNF [127]. BDNF promotes neuronal differentiation and survival, neurogenesis, and brain plasticity and is thus particularly crucial for learning and memory [128]. High fat diet reduces BDNF level in the hippocampus [129], and the impaired hippocampal synaptic plasticity and cognition are possibly through BDNF's effects on dendritic spines [130]. Diet-induced obesity reduces hippocampal expression of BDNF and presynaptic synaptophysin, which are related to an impairment of spatial learning in mice [131].

Although mounting evidence shows that obesity is associated with structural and functional brain changes, the causal link between them requires further investigations. In contrast, the causal link between diet and brain changes is much clearer. The composition of gut microbiota appears to be causally related to obesity [132–134], playing a significant role in body weigh regulation since birth [135, 136]. Gut microbiota plays a key role in childhood obesity and brain development [137, 138]. A comparison of germ-free mice and conventionally reared mice has demonstrated that germ-free mice are leaner and more resistant to diet-induced obesity [139]. Obese and nonobese individuals have different diversity and composition of gut microbiota [140, 141]. As gut microbiota controls energy extraction and storage in the body, significant changes in gut microbiota can result in obesity and insulin resistance [139, 140, 142].

It has been suggested that diet can influence gut microbiota which in turn impacts the brain and behaviors through neural, hormonal, immune, and metabolic pathways [143, 144]. Transplantation of gut microbiota of diet-induced obese mice to lean mice is sufficient to bring about neurobehavioral changes through increasing neuroinflammation and disrupting cerebrovascular homeostasis [145, 146]. Mice consuming high energy diet containing higher percentage of *Clostridiales* and lower expression of *Bacteroidales* have poorer cognitive flexibility [147]. In humans, the *Firmicutes/Bacteroidetes* ratio is positively associated with BMI [148]. Gut microbiota can modulate a range of neurotrophins, such as BDNF and synaptophysin, to affect neural plasticity [149, 150]. Thus, diet changes gut microbiota which influences neurophysiology and neurotrophins, eventually impacting cognition and behaviors.

Previous results have shown that obesity-related brain plasticity alteration is a multifaceted issue, which can inflict permanent harm to individuals in their early ages. Thus, it would be optimal to combat obesity during childhood.

5. Exercise Improves Brain Functions

Exercise can improve physical and cognitive performance, and quality of life in the elderly [151–155]. In humans, those who are highly fit or aerobically trained have greater prefrontal and parietal activations for spatial selection and inhibitory functioning [156]. There is a positive relationship between aerobic fitness and spatial memory which is mediated by hippocampus volume [157]. Aerobic training can increase hippocampal volume of the elderly (with or without mild cognitive impairment) and increases plasma BDNF level in both patients of Alzheimer's disease and healthy controls [158–163]. Regular physical activity is related to better cognition, less cognitive decline, and a lower risk of developing dementia [164, 165]. As young as children, aerobic fitness can predict cognitive performance over time [166]. Besides cognition, higher level of physical activity is related to a reduced white matter hyperintensity burden on motor function in the aged people [167]. BDNF concentration is associated with retention performance of motor skill after learning [168]. Lifelong exercise can preserve white matter microstructure related to motor control and coordination in the elderly [169]. In addition, regular physical activity has long been suggested to be an effective way to improve obesity and related problems [170, 171]. Exercising 5 days per week for 15 weeks can improve executive functions in overweight children [172]. High-intensity physical activity (both aerobic and endurance training) for 4 months improves cognition and oxygen extraction in obese individuals [173].

The effectiveness of exercise may be moderated by exercise intensity and duration, and exerciser's developmental stage [174, 175]. Exercise intensity can be related both to behavioral outcomes and to changes in brain structure and BDNF level. High dose group improves planning more than the low dose group [172]. Greater amount of physical activity in early life is associated with larger prefrontal and hippocampal volumes [176]. Individuals receiving low-intensity exercise, but not high-intensity, show increased BDNF expression [177]. BDNF level depends on exercise intensity [178]; some observe that moderate-intensity exercise is the most effective to promote BDNF in the elderly [179]. Thus, it seems that a moderate intensity of exercise is optimal. In addition to exercise intensity, duration of exercise is also crucial. Tomporowski et al. [180] fail to observe any augmentation of task switching performance after a single bout of moderately intense exercise. In midlife mice, only 4-month (but not 2-month) running training can trigger activation of the antiamyloidogenic, prosurvival, synaptogenic, and neuroprotective pathways [181]. Wheel running for 14 days can increase cell proliferation in the dentate gyrus whereas wheel running for 56 days can additionally facilitate long-term potentiation in this region [182]. These show that a longer duration of exercise favors changes in the brains. Moreover, the developmental stage of exerciser is associated with benefits of exercise. Four-week exercise can improve recognition memory in adult rats, but no such enhancement can be recorded 2 weeks after cessation of training. However, in adolescent rats, the enhancement of recognition memory is preserved [183]. These nicely demonstrate that younger animals benefit more from exercise.

At the neuronal level, physical activity can enhance neurogenesis, neuroadaptation, and neuroprotection though the actions of neurotrophic factors [184–190]. Hippocampal function is restored by physical activity through enhancing the expression of neurotrophic factors to promote neurogenesis, angiogenesis, and synaptic plasticity [191–193]. For example, BDNF level increases with physical activity, particularly regular exercise [194, 195]. It is found that BDNF can stimulate DNA repair to protect cortical neurons against oxidative stress [196]. Short bout of mild exercise for 5 weeks improves both oxygen consumption and long-term spatial learning and memory in aged rats which is associated with hippocampal BDNF level [197]. Following physical activity, hippocampal BDNF level and TrkB receptor activation are increased [198]. The elevated BDNF level in the dentate gyrus is sufficient to induce spatial memory improvement [199]. A week of voluntary exercise is sufficient to increase the activity of tissue type plasminogen activator to facilitate the cleavage of proBDNF into mBNDF [200]. Also, exercise promotes sirtuin 1, stimulates mitochondrial biogenesis, and prevents neurodegeneration [201].

Exercise can be related to structural brain changes [202]. A 7-day exercise intervention can increase gray matter volumes in the motor, somatosensory, association, and visual cortices in rats [203]. Exercising for 6 months reduces default mode network activity in the precuneus [204] while one-year walking increases functional connectivity within the default mode network and frontal executive network [205]. Regular physical activity can reduce proinflammatory and increase anti-inflammatory signaling and reduce oxidative stress in aged animals [206, 207]. Exercise also reduces peripheral risk factors, such as diabetes and cardiovascular diseases which are associated with neurodegeneration [208]. Furthermore, vasculature is altered after exercise. In middle-aged rats, total length and surface area of cortical capillaries are increased

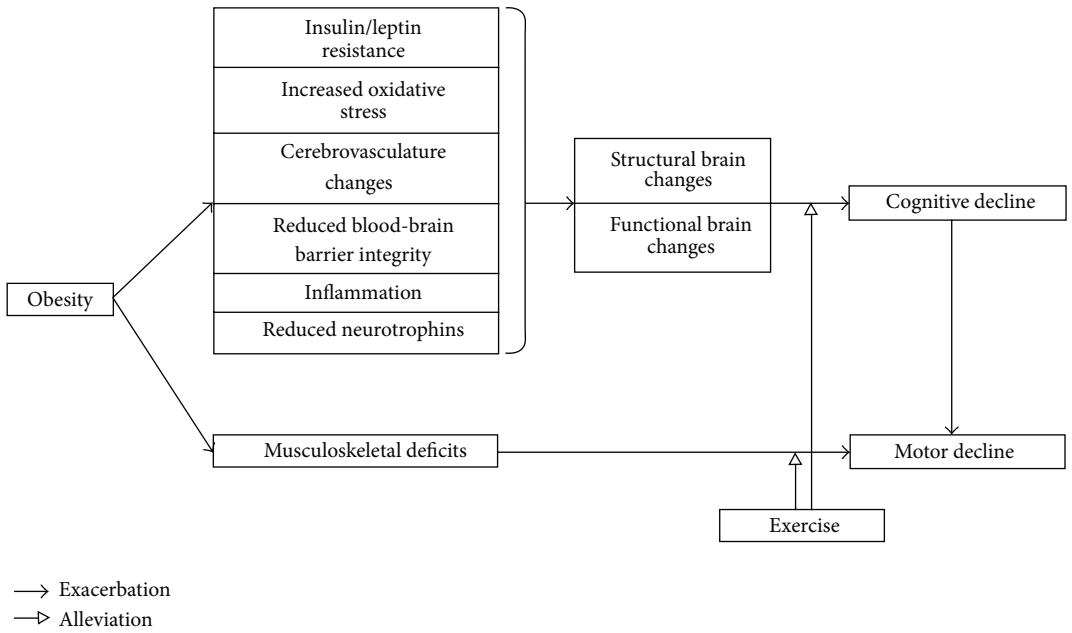

Figure 1: Factors mediating the effects of obesity and exercise on cognition and motor behaviors. Obesity affects cognition mainly through brain changes and influences motor behaviors through degrading the musculoskeletal system. Exercise can alleviate the deleterious effects of the obesity-related mediators on cognition and motor performance.

after running [209]. Aerobic exercise at midlife can improve vascular dysfunctions, astrocyte hypertrophy, and myelin dysregulation associated with sedentary lifestyle [210, 211].

Exercise is associated with a range of improvements in the brain through a range of mechanisms in individuals of different weight statuses (Figure 1). The effectiveness of exercise depends on the training parameters, such as intensity, duration, and developmental stage of exerciser. Previous research results have consistently suggested that moderately intense exercise for a long enough period of time is especially beneficial for young exercisers.

6. Implications and Future Research

More and more people are becoming obese, producing aversive effects on their cognition, motor behaviors, and quality of life [1]. Previous research has suggested that altered brain structure and activation mediate obesity's influences on cognition [17–21], whereas obesity influences the musculoskeletal system to degrade motor performance [59]. As motor performance partly depends on cognitive ability [64], obesity may indirectly contribute to motor deficits through cognitive decline (Figure 1).

Substantial research has shown that obesity affects our cognition and motor behaviors through different mechanisms, possibly through altering brain structure, leptin/insulin regulation, oxidative stress, cerebrovascular function, blood-brain barrier, and inflammation [11, 65–71]. The validity of these proposed mechanisms requires further examinations.

Regular physical activity benefits both cognition and motor behaviors. It is suggested that moderately intense exercise for a long enough period of time seems favorable; however, the training parameter for optimal outcomes remains to be determined. Most of the previous research focuses on aerobic exercise; the efficacy of anaerobic exercise to improve obesity and related dysfunctions is not well understood. More efforts should be devoted to investigate the efficacy of anaerobic exercise, in comparison with aerobic exercise. Moreover, starting exercising in young age is particularly important to protect from neurodegeneration in old age. As childhood obesity is becoming more prevalent [23–25], introducing physical activity intervention in childhood may help children improve obesity and prevent age-related functional decline in old age.

In addition to exercise, leptin replacement therapy, inhaled insulin therapy, and caloric restriction have also been proposed to improve obesity. Leptin is responsible for energy balance and body weight and can affect neurogenesis and brain functions [212]. It enhances immune response and regulates inflammation [212]. It is observed that 18-month leptin replacement therapy increases gray matter concentration and activations in brain regions implicated in hunger and satiation neural circuits [213, 214]. During weight loss, leptin is reduced, facilitating food intake. Leptin therapy helps sustain weight loss [215].

There are insulin disturbances in obese individuals [216, 217]. Insulin resistance plays an important role in obesity and cognitive impairments [218]. It is found that intranasal insulin exerts anorexic effects, promoting satiety and regulating food intake [219, 220]. Inhaled insulin reaches the brain through olfactory nerves and specific receptors in blood-brain barrier to exert its effects [221]. Caloric restriction also improves obesity and reverses deficits in leptin receptor

protein and signaling associated with diet-induced obesity [222]. After 3 months of caloric restriction, serum BDNF increases in overweight and obese individuals [223]. Diet-induced weight loss is related to a decrease in plasma free fatty acid and improvement in episodic memory [224]. Hitherto, the efficacy of leptin replacement therapy, inhaled insulin therapy, and caloric restriction on cognition and motor behaviors is poorly understood, which warrants further verification.

7. Conclusions

Obesity has become a worrying health and social issue. It affects cognition mainly through altering the brain structures and functions [17–21], and motor performance through degrading musculoskeletal system [59]. Obesity can affect brain structure, leptin/insulin dysregulation, oxidative stress, cerebrovascular function, blood-brain barrier, and inflammation [11, 65–71], which are involved in the deterioration of cognitive and motor functions. A host of previous research has suggested that exercise can improve both obesity-related cognitive and motor declines. As more and more people develop obesity in young age, introducing exercise intervention early would result in the greatest benefits.

Disclosure

Chuanming Wang and John S. Y. Chan are co-first authors.

Conflict of Interests

The authors have no conflict of interests to declare.

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Research Article

A Mutation in *DAOA* Modifies the Age of Onset in *PSEN1* E280A Alzheimer's Disease

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We previously reported age of onset (AOO) modifier genes in the world's largest pedigree segregating early-onset Alzheimer's disease (AD), caused by the p.Glu280Ala (E280A) mutation in the *PSEN1* gene. Here we report the results of a targeted analysis of functional exonic variants in those AOO modifier genes in sixty individuals with *PSEN1* E280A AD who were whole-exome genotyped for ~250,000 variants. Standard quality control, filtering, and annotation for functional variants were applied, and common functional variants located in those previously reported as AOO modifier loci were selected. Multiloci linear mixed-effects models were used to test the association between these variants and AOO. An exonic missense mutation in the *G72* (*DAOA*) gene (rs2391191, $P = 1.94 \times 10^{-4}$, $P_{FDR} = 9.34 \times 10^{-3}$) was found to modify AOO in *PSEN1* E280A AD. Nominal associations of missense mutations in the *CLUAP1* (rs9790, $P = 7.63 \times 10^{-3}$, $P_{FDR} = 0.1832$) and *EXOC2* (rs17136239, $P = 0.0325$, $P_{FDR} = 0.391$) genes were also found. Previous studies have linked polymorphisms in the *DAOA* gene with the occurrence of neuropsychiatric symptoms such as depression, apathy, aggression, delusions, hallucinations, and psychosis in AD. Our findings strongly suggest that this new conspicuous functional AOO modifier within the *G72* (*DAOA*) gene could be pivotal for understanding the genetic basis of AD.

1. Introduction

Alzheimer's disease (AD, OMIM 104300), the most common type of dementia, is a neurodegenerative disorder characterized by learning disabilities, cognitive decline, aggression, and short- and long-term memory loss [1]. Mutations in the *Presenilin-1* (*PSEN1*) [2], *Presenilin-2* (*PSEN2*) [3], and *amyloid precursor protein* (*APP*) [4] genes cause early-onset AD (EAOD). A rare mutation (with a minor allele frequency [MAF] of <1%) in *APP* had a protective effect against AD in Icelanders [5], whilst a rare mutation in the *Phospholipase D family member 3* (*PLD3*) gene segregates in two families with late-onset AD (LOAD) and doubles the risk of AD in European and African American cases/control samples [6], but this association failed to replicate in a subsequent study [7]. Likewise, a mutation in the *Triggering receptor expressed on myeloid cells 2* (*TREM2*) gene was found to double the risk of AD in two independent case/control samples [8], associated in a family with frontotemporal lobar degeneration [9]. TREM2 is also overexpressed in brain tissue from individuals with AD [9].

Over the last 30 years, our group has studied the world's largest multigenerational pedigree in which a mutation in the *PSEN1* gene, also known as the *PSEN1* p.Glu280Ala E280A mutation (often referred to as the *Paisa* mutation), cosegregates with EOAD [2, 10]. This pedigree originated as a consequence of a founder effect [11] initially traced to 1783 [12] and localizes in a homogeneous environment [12, 13].

These two factors, along with the presence of exhaustive and detailed medical records of several hundred individuals, make this pedigree a powerful tool in genetic research [14–16]. Genome sequencing analysis successfully tracked the most common ancestor and the first mutation event for the E280 mutation to 10 and 15 generations ago, respectively [17].

To date, more than 5,000 individuals are descendants of the original founder, 1,784 of whom were enrolled to participate in comprehensive ongoing clinical monitoring. Of those, 459 mutation carriers and 722 noncarriers have been genotyped. Although the median age of onset (AOO) of AD in these individuals is ~49 years [95% CI 49-50], the broad spectrum of the AOO of dementia symptoms can be in the range of ~30–80 years [13, 16].

We previously identified both known and novel loci genome-wide significantly associated with AOO in AD, including *D-amino acid oxidase activator* (*DAOA*; rs778296, $P = 1.58 \times 10^{-12}$), *Homo sapiens CD44 molecule* (*CD44*; rs187116, $P = 1.29 \times 10^{-12}$), *Gremlin 2, DAN family BMP antagonist* (*GREM2*; rs12129547, $P = 1.69 \times 10^{-13}$), *Nephronophthisis 1 (juvenile)* (*NPHP1*; rs10173717, $P = 1.74 \times 10^{-12}$), *Homo sapiens* Ca^{++}*-dependent secretion activator 2* (*CADPS2*; rs3757536, $P = 1.54 \times 10^{-10}$), *Homo sapiens clusterin associated protein 1* (*CLUAP1*; rs1134597, $P = 1.12 \times 10^{-8}$), and *Homo sapiens exocyst complex component 2* (*EXOC2*; rs2804737, $P = 3.28 \times 10^{-6}$) [18]. Although the AOO modifier effect of the *NPHP1* gene has been confirmed in a Caribbean population with AD and the G206A mutation in *PSEN1* [19], the functional assessment of the remaining variants was yet to be performed.

In this paper, we present the targeted analysis of functional exomic variants harboured in those genes reported as potential modifiers of the AOO of AD by a genome-wide association study (GWAS) [18]. We found that an exonic missense mutation in the *DAOA* (rs2391191, Arg30Lys, $P = 1.94 \times 10^{-4}$, $P_{FDR} = 9.34 \times 10^{-3}$) gene modifies the AOO in *PSEN1* E280A AD. Furthermore, nominal associations in *CLUAP1* (rs9790, Arg235Trp, $P = 7.63 \times 10^{-3}$, $P_{FDR} = 0.1832$) and *EXOC2* (rs17136239, Gln201Arg, $P = 0.0325$, $P_{FDR} = 0.391$) were also found. Clinical, biological, and mouse models evidence suggest that these functional coding variants are important players in shaping the susceptibility to AD, opening new windows towards outlining the genetic basis of this devastating neurodegenerative disease.

2. Methods

2.1. Subjects. Sixty patients with AD carrying the Paisa mutation, and displaying an extreme AOO, were selected from our clinical study for whole-exome genotyping (36 women [60%] and 24 men [40%]) [13]. The mean AOO of AD was 47.8 ± 6.4 years. No difference in the average AOO in AD was found by gender (female: 48.0±7.02; male: 47.4±5.52, $P = 0.702$) (Figure 1(a), top). A total of 49 patients (28 women [57%] and 21 men [43%]) had an AOO of AD below 48 years and *ad hoc* classified as EOAD, whilst the remaining 11 individuals were *ad hoc* classified as LOAD [18]. As intended, the average AOO was significantly different between EOAD and LOAD patients (EOAD: 45.1 ± 2.22, LOAD: 59.4 ± 6.15, $P < 1.41 \times 10^{-5}$) (Figure 1(a), middle). Years of education ranged from 0 to 19 years. Four patients (7%) never attended school, 30 (50%) completed primary school (grades 1 to 5), 22 (37%) completed high school (grades 6 to 11, inclusive), and only 4 (6%) had tertiary education. No difference was found in AOO of AD across education groups ($F_{3,56} = 1.487$, $P = 0.228$) (Figure 1(a), bottom).

2.2. Whole-Exome Genotyping. Genomic DNA from 60 participants was whole-exome genotyped by the Australian Genome Facility (Melbourne, VIC, Australia), an Illumina Certified Service Provider for the Infinium Genotyping Service. Briefly, DNA was whole-genome amplified, fragmented, hybridized, fluorescently tagged, and scanned [20]. Whole-exome genotyping was conducted using Illumina's HumanExome 12v1_A BeadChip. This chip covers regions with putative functional exonic variants selected from exome- and whole-genome sequences of >12,000 individuals. The exonic content consists of >250,000 markers representing diverse populations (including European, African, Chinese, and Hispanic individuals) in addition to common conditions (such as type 2 diabetes, cancer, and metabolic and psychiatric disorders). In order to test genotyping reliability and quality, one individual was duplicated. The identity by descent (IBD) matrix between all pairs of individuals was used for quality control and for subsequent analyses concerning the mixed model (see below). Entries of the IBD matrix contain the probability that a particular allele is inherited from a common ancestor [21].

2.3. Genetic/Statistical Analysis

2.3.1. Quality Control and Filtering. Genotypes were extracted using the Genotyping module of Illumina's GenomeStudio v2010.3 (with the default settings) and the Illumina HumanExome 12v1_A manifest cluster file. Samples with calls below Illumina's expected 99% single nucleotide polymorphisms (SNPs) call rates were excluded. Genotype files were processed in Golden Helix SNP and Variation Suite (SVS) 8.0.2 (Golden Helix, Inc., Bozeman, MT, USA) using the GenomeStudio DSF Plugin. Golden Helix SVS is an integrated collection of analytic tools for managing, analyzing, and visualizing multifaceted genomic and phenotypic data.

For replication purposes, only variants located in the top 30 chromosomal regions reported as potential modifiers of the AOO in patients AD carrying the Paisa mutation [18] were included for further analysis. Marker exclusion criteria included (i) deviations from the Hardy-Weinberg equilibrium with $P < 2 \times 10^{-7}$ (0.05/250,000 markers) in both cases and controls (a stringent criterion to avoid the exclusion of any causal variant of major effect), (ii) a minimum genotype call rate of 90%, (iii) the presence of one or more than two alleles, and (iv) a MAF < 1%. Genotype and allelic frequencies were estimated by maximum likelihood.

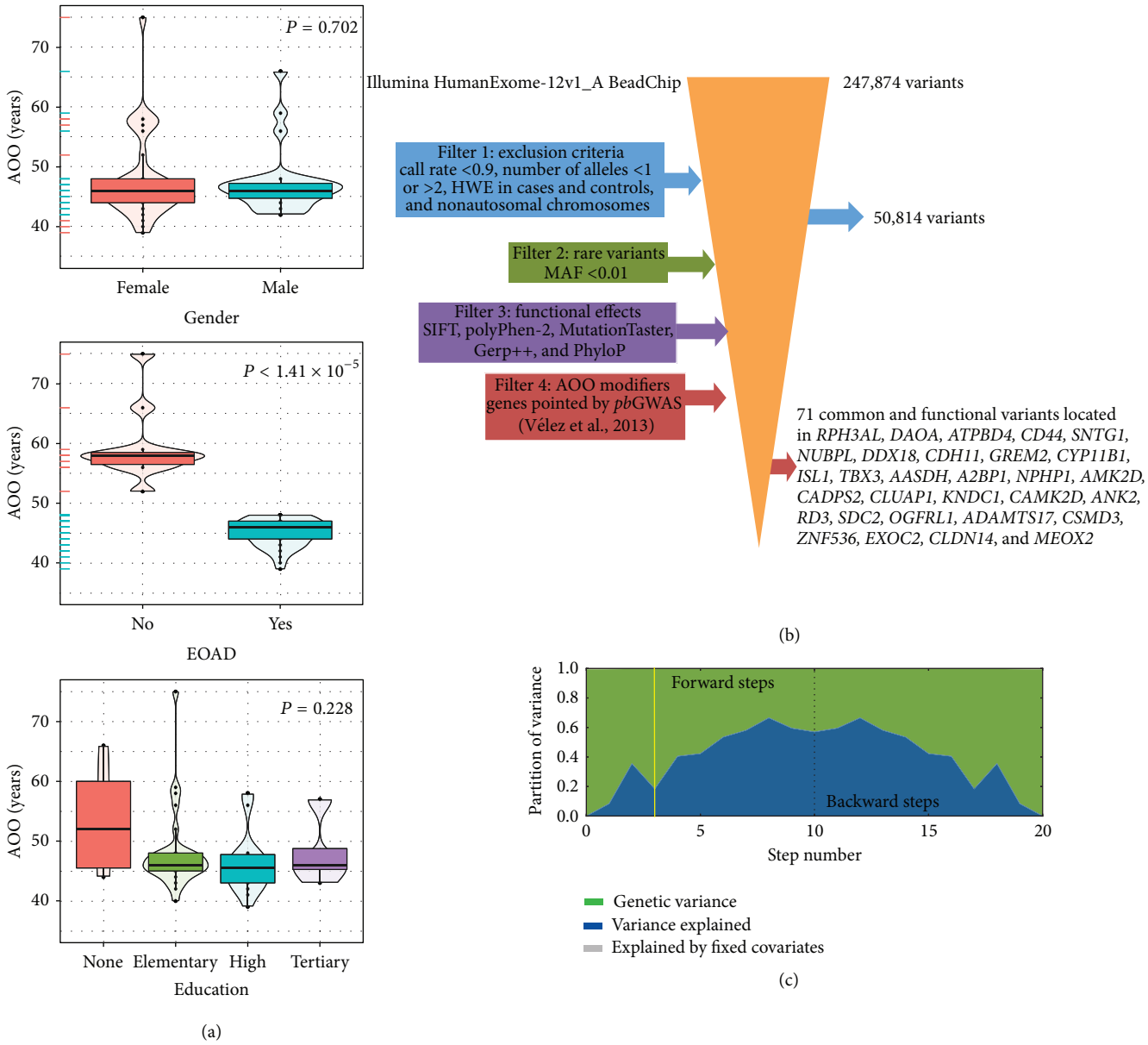

Figure 1: (a) Box- and violin-plots for the AOO of AD by gender (top), early-onset (middle), and level of education (bottom) in 60 patients carrying the *PSEN1* E280A mutation. The associated *P* value after testing for differences in the average AOO is shown. AOO: age of onset; AD: Alzheimer's disease; EOAD: early-onset Alzheimer's disease. (b) Filtering workflow of exonic variants leading to the selection of 71 variants harboured in genes associated with modifiers of the AOO of AD in carriers of the *PSEN1* E280A mutation as reported by Vélez et al. [18]. Abbreviations as in (a). (c) Partition of phenotypic variance for each forward inclusion (steps 1 to 10) and backward elimination (10 steps after the dotted line). The yellow vertical line marks the model selected based on the lowest Bayesian Information Criterion (BIC).

2.3.2. Filtering and Classification of Functional Variants. Exonic variants with potential functional effect were determined using the functional prediction information available in the dbNSFP_NS_Functional_Predictions GRCh_37 annotation track. This filter uses SIFT [22], PolyPhen-2 [23], MutationTaster [24], Gerp++ [25], and PhyloP [25] and is implemented in the SVS Variant Classification module. Variants were classified based on their potential effect on genes according to their position in a gene transcript, and those variants in coding exons were subsequently classified according to their potential effect on the gene's protein structure. This method gives insight into which variants are most likely to have functional effects.

2.3.3. Genetic Analysis of Exonic Variants. Single- and multilocus additive linear mixed-effect models (LMEMs) [26–28] were fitted to test the association of these variants to AOO of AD. The advantage of these models is the inclusion of both fixed (sex and years of education) and random effects, the latter to account for kinship effects by including

the IBD matrix. The single-locus LMEM assumes that all loci have a small effect on the trait, whilst multilocus LMEMs assume that several loci have a large effect on the trait. In a single-locus model, the association between the variant of interest and the disease trait is tested after covariates and genetic stratification are controlled for. Conversely, in a multilocus model the association is tested after covariates, genetic stratification, and the effect of the remaining $m - 1$ variants are controlled for. These recently emerging methods have been proven to be more powerful than existing methods [28]. Furthermore, this family of models allows handling of confounding effects and accounts for loci of small- and large-effect in structured populations with a small computational burden [28]. After the estimation process was finished, the coefficients $\widehat{\beta}_1, \widehat{\beta}_2, \ldots, \widehat{\beta}_m$ from the linear mixed-effects model were extracted and a hypothesis test of the form $H_{0,i}$: $\beta_i = 0$ versus $H_{1,i}$: $\beta_i \neq 0$ was performed for the ith exonic variant to obtain the corresponding P value ($i = 1, 2, \ldots, m$). Thus, the collection $P_1, P_2, \ldots, P_m$ of P values was corrected for multiple testing using the false discovery rate (FDR) [29] and a method based on extreme-values theory [30]. Because the tests of hypothesis being performed are of the same type, correction was performed on the resulting m P values only [29, 30]. Exonic variants significantly associated with the AOO of AD were determined based on these derived P values.

3. Results

3.1. Quality Control. A total of 247,874 variants in the Illumina's HumanExome 12v1_A BeadChip were submitted to quality control. In the first filter, 50,814 variants with call rate > 0.9, in Hardy-Weinberg equilibrium in both cases and controls and located on autosomal chromosomes, were kept. This number was reduced to 71 common variants with potential functional effects at the end of the filtering process (Figure 1(b)). These resulting common variants are harboured in chromosomal regions reported as modifiers of the AOO in *PSEN1* p.Glu280Ala E280A AD, as reported by Vélez et al. [18].

3.2. Exonic Associated Variants. Multilocus additive LMEMs including all 71 common variants located in genes modifying the AOO in patients with *PSEN1* p.Glu280Ala E280A AD were fitted. Based on the Bayesian Information Criterion (BIC), a LMEM with three steps in the forward/backward selection algorithm [28] was selected (BIC = −50.8). In this model, the pseudoheritability (defined as the proportion of inheritance explained by the random effects) was 0.9987, whilst the proportion of genetic variance explained was ~ 20% (Figure 1(c), yellow vertical line). We found that variant rs2391191 (UCSC GRCh37/hg19 coordinates) is significantly associated with AOO in our sample of 60 individuals with AD carrying the Paisa mutation ($P = 1.94 \times 10^{-4}$, $P_{\text{FDR}} = 9.34 \times 10^{-3}$). Located in position 106,119,446 of chromosome 13, this is a missense variant (Arg30Lys) in the *DAOA* gene (NM_172370). Two more exonic variants were found to be nominally associated with the AOO in patients with *PSEN1* E280A AD: rs9790 ($P = 7.63 \times 10^{-3}$, $P_{\text{FDR}} = 0.1832$) mapping to chr 16: 3,586,230 (UCSC GRCh37/hg19 coordinates) and corresponding to a missense variant (Arg235Trp) in the *CLUAP1* gene (NM_015041) and rs17136239 ($P = 0.0325$, $P_{\text{FDR}} = 0.391$) which maps to chr 6: 656,343 (UCSC GRCh37/hg19 coordinates) and corresponds to a missense variant (Gln201Arg) in *EXOC2* (NM_018303).

4. Discussion

We previously reported that variants within or close to the *DAOA*, *CLUAP1*, and *EXOC2* genes were identified as AOO modifiers of AD in carriers of the *PSEN1* E280A mutation [18]. Here, we report that a common functional exonic variant in *DAOA* modifies the AOO of AD in those patients. Although further studies are required to replicate this finding in other populations, this result suggests a potential genetic interaction [31] between *PSEN1* and *DAOA*, similar to what has been shown in genes involved in cholesterol, amyloid, inflammation, and oxidative stress in sporadic [32], late-onset [33], and familial AD [34].

The *DAOA* gene, also known as *G72*, is located in the 13q33.2 chromosomal region, spans 25,168 bp (UCSC GRCh37/hg19 coordinates), and its expression is enriched in the brain, spinal cord, and testis. In mice, G72 has been found to be overexpressed in testis and cerebral cortex, with low to no expression in other tissues [35]. *DAOA*, which has typically been associated with bipolar disorder (BD) and schizophrenia (SZ), encodes a protein that may act as an activator of the DOA (*D-amino acid oxidase*) enzyme, which degrades the gliotransmitter D-serine, a potent activator of N-methyl-D-aspartate (NMDA) type glutamate receptors [36]. Polymorphisms in *DAOA* have been associated with the occurrence of neuropsychiatric symptoms such as depression, apathy, aggression, delusions, hallucinations, and psychosis in AD [37, 38]. In particular, the development of psychotic symptoms has been attributed to a similar psychosis-modifier gene mechanism to that in SZ because of the cytokine pathway disruption in both diseases [39, 40].

NMDA receptors (NMDARs) are glutamate-gated cation channels with high calcium permeability, critical for the development of the central nervous system (CNS), generation of rhythms for breathing and locomotion, and the processes underlying learning, memory, and neuroplasticity [41–44]. NMDARs regulate the functional and structural plasticity of individual synapses, dendrites, and neurons by activating specific calcium-dependent signaling cascades [44–46]. Specifically, both synaptic strengthening and weakening processes are mediated by Ca^{2+} influx through NMDARs [44]. Evidence in mouse models suggests that adult mice benefit from the genetic enhancement of the NMDAR function as it improves memory, but that blocking the NMDAR in the brain compromises learning and spatial memory as a consequence of the impairment of synaptic plasticity [41, 43, 46–49]. Furthermore, abnormal expression levels and altered NMDAR function have been implicated in numerous neurological disorders, including AD [44, 45], and therefore considered an important therapeutic target in this neurodegenerative disease [43–45]. In fact, a partial NMDAR

antagonist, memantine, was approved to treat moderate to severe AD in the US and Europe [50, 51]. However, the success of memantine and other NMDARs has been limited in the clinical setting due to their low efficacy and side effects [44, 52, 53].

Two more exonic variants, one in *CLUAP1* (NM_015041) and one in *EXOC2* (NM_018303), were found to be nominally associated with the AOO in patients with the *PSEN1* E280A AD. The *CLUAP1* gene spans 38,125 bp in the 16p13.3 chromosomal region and interacts with *APP*, the *Homo sapiens clusterin* (*CLU*),and the *Melanoma associated antigen 11* (*MAGEA11*) genes [54]. Gene ontology analyses suggest an important role of *CLUAP1* in synaptic growth at neuromuscular junction, neuron remodelling, exocytosis, axon midline, and smooth endoplasmic reticulum calcium ion homeostasis. Mouse models support the role of Cluap1 in ciliogenesis due to the concentration of p75 neurotrophin receptors in the primary cilia membranes [55]. In humans, cilia are involved in numerous cellular activities [56] and have been suggested to impact cognitive deterioration in AD as a consequence of the neurogenesis process occurring in the hippocampus (which is necessary for new memory encoding) [57]. Subsequently, novel therapeutic approaches to AD, especially at the early stage of its development, have been outlined [57].

The *EXOC2* gene encodes a protein member of the exocyst complex. This complex, triggered in many ways by Ca^{2+} [58], is essential for tying exocytic vesicles to the plasma membrane [59]. In mice, higher total presynaptic mitochondrial volumes are associated with higher levels of exocytosis in stimulated hippocampal synaptosomes [47]. Furthermore, weighted gene coexpression analysis of posterior cingulate (PC) astrocytes in AD showed that *EXOC2* was part of the largest coexpressed modules, providing evidence that brain immunity and mitochondrial function in PC astrocytes are perturbed in AD [60]. These findings correlate with other studies suggesting an important role of astrocytes in AD, particularly in the earliest neuronal deficits [61], and their contribution to the neuroinflammatory component of neurodegeneration during latter stages of the disease [62].

5. Conclusions

Here we present a follow-up of our GWAS study linking several loci to the AOO of AD [18] in the world's largest genealogy segregating EOAD. Previous studies have linked polymorphisms in the *G72* (*DAOA*) occurrence of neuropsychiatric symptoms in AD, and this study confirms the existence of an AOO modifier mutation in the *DAOA* gene, a usual suspect associated with shaping the natural history of AD.

Disclosure

The sponsor of the study has no role in the study design, data collection, data analysis, data interpretation, or writing of the reports. Jorge I. Vélez, Francisco Lopera, and Mauricio Arcos-Burgos have full access to all the data in the study. Jorge I. Vélez and Mauricio Arcos-Burgos are responsible for submitting this work for publication. Jorge I. Vélez is a doctoral student at ANU; some of this work is to be presented in partial fulfilment of the Ph.D. degree requirements.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Effects of Leptin Replacement on Neural Plasticity

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Leptin, an adipokine synthesized and secreted mainly by the adipose tissue, has multiple effects on the regulation of food intake, energy expenditure, and metabolism. Its recently-approved analogue, metreleptin, has been evaluated in clinical trials for the treatment of patients with leptin deficiency due to mutations in the leptin gene, lipodystrophy syndromes, and hypothalamic amenorrhea. In such patients, leptin replacement therapy has led to changes in brain structure and function in intra- and extrahypothalamic areas, including the hippocampus. Furthermore, in one of those patients, improvements in neurocognitive development have been observed. In addition to this evidence linking leptin to neural plasticity and function, observational studies evaluating leptin-sufficient humans have also demonstrated direct correlation between blood leptin levels and brain volume and inverse associations between circulating leptin and risk for the development of dementia. This review summarizes the evidence in the literature on the role of leptin in neural plasticity (in leptin-deficient and in leptin-sufficient individuals) and its effects on synaptic activity, glutamate receptor trafficking, neuronal morphology, neuronal development and survival, and microglial function.

1. Introduction

Leptin is a 16-kDa hormone with cytokine-like actions (i.e., adipokine or adipocytokine), synthesized and secreted mainly by the white adipose tissue. As one of the most abundant adipokines, leptin has crucial effects on the regulation of food intake and energy balance [1]. Since its discovery in 1994, many additional actions have been described, with fundamental roles in lipid and glucose homeostasis, immunity, inflammation, bone physiology, reproduction, regulation of thyroid, growth hormone and adrenal axes, and tissue remodeling. Those actions have been identified mainly through animal models of leptin deficiency (namely, the *ob/ob* and the *db/db* mice), but also through studies carried out in humans with leptin deficiency: patients with lipodystrophy syndromes, hypothalamic amenorrhea, and congenital leptin deficiency (CLD) due to mutations in the leptin gene [2].

Humans with leptin deficiency develop metabolic dysfunctions such as increased insulin resistance, hyperglycemia, dyslipidemia, endocrine disruptions, and fatty liver disease. In addition, morbid obesity, impaired cognitive development, and potentially lethal T-cell hyporesponsiveness have been reported in patients with mutations in the leptin gene [3].

More recently, animal and human studies have shown that leptin has remarkable effects on neural plasticity and cognition [4]. In humans with leptin deficiency, leptin replacement therapy (LRT) has led to time-dependent increases in gray matter concentration in diverse brain regions (including the hippocampus) and to changes in activation of regions traditionally linked to hunger, satiety, and reward. Moreover, improvements in neurocognition have been reported in isolated cases [5]. In normoleptinemic humans, circulating leptin levels have also been associated with alterations in cognition [6]. Furthermore, studies suggest that leptin is neuroprotective [7], and low leptin levels may play a role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) [8].

In this paper, the effects of leptin on the structure and function of the central nervous system will be described. The literature presenting the association (or lack therefore) between leptin and AD will be reviewed, and the future directions for studies on the role of leptin replacement in neural plasticity will be discussed.

2. Biology of Leptin

Leptin has structural homology with long-chain helical cytokines including IL-6, IL-11, IL-12, and oncostatin M. Leptin has tridimensional characteristics of a four-helical bundled cytokine, similar to those cytokines that activate the Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) pathway [9]. In fact, the structural similarities that leptin shares with the IL-6 cytokine family led to functional *in vitro* signaling studies, which proved that leptin does in fact activate the JAK2-STAT3 pathway, although STAT1, STAT5, and STAT6 may be activated by leptin as well [10].

Its receptor, Ob-R (or Lep-R), is structurally similar to members of the class I cytokine receptor (gp130) superfamily. There are at least six different isoforms of the leptin receptor in rat: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re, and Ob-Rf; all of them are products of six alternatively spliced forms of the Ob-R gene [11]. Ob-Rb is the only long form, containing a long cytoplasmic region with several motifs required for signal transduction and activation of the intracellular pathways. There are four truncated (short) forms (Ob-Ra, Ob-Rc, Ob-Rd, and Ob-Rf); Ob-Ra is regarded as a leptin transporter across the blood-brain barrier (BBB) and a leptin degrader, and Ob-Re is a secreted form lacking intracellular and transmembrane domains, serving as plasmatic leptin-binding protein. All isoforms (except Ob-Rb) share the same identical extracellular ligand-binding domains, differing at the C terminus. All these isoforms are involved in the mediation of leptin's actions in peripheral organs and in the brain.

The murine and human leptin receptors are highly similar in amino acid sequences for both the extracellular (78% identity) and intracellular domains (71% identity) [12]. In humans, a soluble isoform (sOb-R, homologous to murine Ob-Re) and four different membrane-anchored Ob-R isoforms have been described: Ob-R219.2, homologous to murine Ob-Ra; Ob-Rfl, homologous to murine Ob-Rb; Ob-R219.3, homologous to murine Ob-Rc; and Ob-R219.1, homologous to murine Ob-Rd [13]. No Ob-Re transcript has been found in humans: in rodents, to create Ob-Re, the splice site at the 3′-end of exon 14 is skipped, leading to the transcription of a stop codon and a polyadenylation signal; in humans, the sequence 5′ of exon 14 does not have a polyadenylation signal. Instead, Ob-Re is generated by proteolytic cleavage. Ob-Rf has only been found in rat.

Besides activating the JAK2/STAT pathway, leptin controls other key signaling pathways: mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase- (ERK) 1/2, and phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/forkhead box protein O1 (FoxO1) pathways [24]. The activation of MAPK/ERK pathway is believed to be the main mechanism involved in the regulation of cell cycle and proliferation [25]; the PI3K pathway is an acute phase contributor of inflammation and is also involved in the phosphorylation of insulin receptor substrate (IRS), important for glucose homeostasis. Leptin's intracellular pathways are downregulated by proteins such as suppressor of cytokine signaling 3 (SOCS3), protein tyrosine phosphatase 1B (PTP1B), and src homology-2-containing protein tyrosine phosphatase 2 (SHP2). The upregulation of genes synthetizing such proteins is the main contributor to leptin resistance [26].

In order to elicit central actions, leptin must cross the BBB through a saturable, passive transport across the barrier. The leptin receptor isoforms Ob-Ra and Ob-Rc have been shown to mediate BBB transport of leptin, and dysfunctional receptors may lead to leptin resistance. More recently, it has been shown that the tanycytes in the median eminence take up blood-borne leptin in an Ob-Rb dependent manner, constituting a route for entry into the hypothalamus [27]. Hypertriglyceridemia may also be another contributor to leptin resistance, as high triglyceride levels decrease leptin transport across the BBB [28].

An important function of leptin is to regulate energy expenditure and food intake, by its actions in the arcuate nucleus (ARC) of the hypothalamus. In this area, leptin binds to its receptors, expressed in two different neuronal populations: the ones that express agouti-related peptide (AgRP) and neuropeptide Y (NPY) and those that express the peptide cocaine and amphetamine-related transcript (CART) and the peptide pro-opiomelanocortin (POMC). Leptin exerts anorexigenic effects by inhibiting the AgRP/NPY neurons and by stimulating the POMC/CART neurons [1].

3. Leptin Replacement Therapy

Studies evaluating LRT in patients with leptin deficiency due to mutations in the *LEP* gene allow the understanding of the physiological effects of its replacement in leptin-sensitive humans not previously exposed to the endogenous adipokine. In humans, leptin deficiency is observed in cases of patients with lipodystrophy syndromes, hypothalamic amenorrhea, and congenital leptin deficiency (CLD) due to mutations in the leptin gene [2]. Several trials of LRT in such patients have been reported in the literature, mostly evaluating the metabolic and endocrine effects of LRT. However, some studies have evaluated the central effects of LRT.

The current presentation of leptin that is available for human therapy is recombinant methionyl human leptin (metreleptin, Myalept, Aegerion Pharmaceuticals, Inc.). In the US, Myalept is available only through the Myalept Risk Evaluation and Mitigation Strategy (REMS) Program, under which prescribers must be certified with the program by enrolling in and completing training. Metreleptin is composed by the 146 amino acids of the mature form of human leptin, with the addition of a methionyl residue at the N-terminal end. Myalept has been recently approved by the FDA for the treatment of congenital or acquired generalized lipodystrophy (non-HIV-related), but it has also been trialed in patients with the partial forms of the disease (approval depending on results from more trials evaluating safety and effectiveness). The recommended starting dose varies by gender and body weight, to a maximum daily dose of 0.13 mg/kg/day (if body weight $\leq$ 40 kg) and 10 mg/day (if body weight > 40 kg). Metreleptin is preferably administered once daily at the same time every day, subcutaneously. It is eliminated unmetabolized via renal clearance, and its

most common reported adverse reactions (≥10%) include headache, hypoglycemia, decreased weight, and abdominal pain. Autoimmune disorder progression (autoimmune hepatitis and membranoproliferative glomerulonephritis) and T-cell lymphoma have been reported in patients with lipodystrophy being treated with metreleptin [29]. Also, hypersensitivity reactions (e.g., urticaria or generalized rash) have been reported [30]. It is contraindicated for patients with general obesity not associated with congenital leptin deficiency and hypersensitivity to metreleptin. Its safety in pregnant women is unknown. In nursing women, either metreleptin therapy or nursing should be discontinued.

Anti-metreleptin antibodies have been identified in nearly all lipodystrophy patients (>95%) treated with metreleptin from two NIH studies (NIH Studies 991265 and 20010769) and Study FHA101 (sponsor-initiated) [30]. The effects of those antibodies have not been well characterized yet, but they can have neutralizing activity and lead to loss of metabolic control by inhibiting leptin action [31, 32].

4. Effects of Leptin Replacement Therapy in the Brain of Patients with Mutations in the Leptin Gene

Cases of CLD caused by mutations in the leptin gene are rare: to date, a total of 38 individuals of Turkish ($n = 6$) [5, 33–35], Pakistani ($n = 27$) [31, 36–40], Austrian ($n = 1$) [41], Egyptian ($n = 2$) [14], Russian ($n = 1$) [42], and Indian ($n = 1$) [43] origins, harboring different mutations in the leptin gene, have been reported. Physiological doses of metreleptin have been evaluated in the treatment of CLD in patients of Turkish, Pakistani, and Austrian background, with remarkable effects on body composition, metabolism, endocrine and immune systems, brain, and behavior. Since leptin plays crucial roles in the immune system, many untreated affected individuals die during childhood due to infections and sepsis. In those cases, leptin therapy may be lifesaving [44].

For patients with CLD, the usual initial dose of metreleptin is 0.02–0.04 mg/kg/day, calculated to achieve 10% of predicted serum leptin concentration (based on pharmacodynamic and pharmacokinetic data from AMGEN Inc., Thousand Oaks, California, USA). Dose remains the same if weight reduces or stabilizes. If weight increases over two consecutive months, the dose is increased to achieve 20%, and subsequently 50%, 100%, and 150% predicted serum leptin concentrations. Dose can be reduced if excessive weight loss occurs [2].

Studies on these patients while on and off LRT have shown that its effects in brain plasticity are remarkable. In Turkish adults with a Arg105Trp missense mutation, 18 months of LRT led to relative gray matter concentration increases in areas associated with regulation of hunger or motor control in human subjects, namely, the frontal cortex (primarily in the left anterior cingulate gyrus), left inferior parietal lobule, and left cerebellum [45]. Peak or maximum effect within significant clusters of voxels were observed in the left anterior cingulate gyrus, left inferior parietal lobule, and left cerebellum, at the same stereotaxic coordinates in scans acquired a year before (after 6 months of LRT), with fewer voxels at 18 months. No significant effect occurred in the hypothalamic region, where leptin determines its effects on food intake. Annual withholding of replacement for 11–36 days per year over 3 years (mean 28.6 days/year) reversed this effect in the left anterior cingulate gyrus and cerebellum with larger extent, and in the left inferior parietal lobule (not significant). Furthermore, short-term treatment reinitiation for 11–22 days/year (mean 16.1 days/year) did not lead to recovery of gray matter concentration in the three expected locations but caused an unexpected increase in gray matter concentration in the posterior half of the left thalamus, particularly the pulvinar nuclei, which are areas implicated in neural circuits regulating food-seeking through relay of taste [15]. The gray matter changes in the cerebellum were directly caused by leptin, whereas the changes in the anterior cingulate gyrus and in the inferior parietal lobule were explained by its effects on body mass index (BMI). Furthermore, an unexpected negative correlation between BMI and gray matter structure in the right inferior temporal gyrus was observed, and a positive correlation between duration of leptin supplementation and gray matter structure in the right hippocampus was also observed [15], in concordance with previous studies showing that leptin levels are positively correlated with hippocampal structure and function [46], which may be related to leptin's effects on memory.

In functional magnetic resonance imaging (MRI) studies, leptin replacement reduced activation of regions linked to hunger (insula, parietal, and temporal cortex) and enhanced activation of regions linked to inhibition and satiety (prefrontal cortex, mainly middle, superior, and medial frontal gyri, and regions linked to satiety and inhibition of high-calorie food intake) [16], as well as the posterior lobe of the cerebellum, the brain region with the highest concentration of leptin receptors [17]. When LRT was withheld, activity was greater in the insula (the primary gustatory cortex) and other temporal/parietal regions (involved in the sensation of hunger), as well as occipital and limbic regions. These results show that leptin has extrahypothalamic effects in the regulation of food intake, reversibly altering neural structure and function and modulating plasticity-dependent brain physiology in response to food cues. Although the cerebellum is not traditionally regarded as an area responsible for the control of food intake, it may be involved in the suppression of motivational food-seeking, when leptin-sufficient individuals are not as hungry. Moreover, in positron emission tomography studies, we have shown that resumption of leptin treatment, after long-term replacement and short-term removal, does not significantly increase striatal D2/D3 receptor availability [21].

In a 15-year-old female Austrian patient, acute leptin therapy did not reduce activity in the hunger-related regions we have previously reported. In functional MRI studies, after 3 days of LRT, Frank et al. observed the activation of reward- and food-processing areas (ventral striatum and the orbitofrontal cortex, resp.). Acute and long-term (6 months of LRT) activation differences were observed in the amygdala and substantia nigra/ventral tegmental area (both decreased) and in the orbitofrontal cortex (increased). When

comparing responses to pictures showing high-calorie versus low-calorie food, the authors observed that the activation of the hypothalamus to high-calorie pictures was decreased over time, whereas low-calorie stimuli led to increased activation of the hypothalamus [18]. In their 1- and 2-year follow-up study, the long-term effects in the amygdala and in the orbitofrontal cortex were sustained, with a decrease of the frontopolar cortex activity. Long-term effects in the hypothalamus showed an assimilating pattern for the response to high- versus low-calorie food, and hedonic regions showed only acute effects after 3 days of LRT [19].

Functional MRI studies carried out in patients of Pakistani background showed that the leptin-deficient state was associated with activation in the anteromedial ventral striatum (nucleus accumbens and caudate nucleus) and posterolateral ventral striatum (putamen and globus pallidus) and 7 days of LRT reduced activation in the nucleus accumbens-caudate and putamen-globus pallidus regions [20].

The procognitive effects of leptin were observed in the youngest Turkish male patient, who showed improvements of several subtests within neuropsychological functioning tests [5]. Neurocognitive assessments (Differential Ability Scales, a measure of general verbal and nonverbal functioning; and selected subtests from the NEPSY, a measure of neuropsychological functioning in children) were conducted at ages 5 (leptin-naïve state), 6, and 7 (on LRT). The patient's pretreatment Differential Ability Scales (DAS) verbal, nonverbal, preacademic, and short-term memory cluster scores were lower than the scores for age-matched controls. However, LRT was followed by an upward trend in development, with scores generally normalizing at age 7. Similar upward trend was observed for scores in the NEPSY visual-spatial and language-memory subtests [5].

5. Effects of Leptin Replacement Therapy in the Brain of Patients with Lipodystrophy

Patients with lipodystrophy share some of the clinical manifestations of CLD, due to low/absent blood leptin levels caused by the generalized or partial absence of subcutaneous adipose tissue. A recent meta-analysis has shown that LRT decreases fasting glucose, HbA1c, triglycerides, total cholesterol, liver volume, and aspartate aminotransferase levels in patients with LS not associated with highly active antiretroviral therapy use [47]. Unfortunately, no structural studies have been conducted in lipodystrophy patients; the only functional study in lipodystrophy patients showed that LRT increased food-related neural activity in the orbitofrontal cortex and suppressed activity in the amygdala, hippocampus, insula, caudate, and putamen, under postprandial conditions (which has little involvement in the regulation of neural activity while fasting) [22].

6. Effects of Leptin Replacement Therapy in Patients with Acquired Leptin Deficiency

The structural and functional effects of LRT have also been evaluated in three women with acquired leptin deficiency (defined as hypoleptinemia for at least 6 months, coincident with strenuous exercise and/or low body weight, without any significant comorbid medical conditions, including eating disorders). In hypoleptinemic women, brain structure and response to visual stimuli while fasting were similar to those of normal controls. In the fed state, participants showed increased activation in precuneus, insula, and dorsolateral prefrontal cortices and decreased activation in insula in response to viewing food. After 1 week of LRT, fasting hypoleptinemic women showed increased activation in bilateral insula, dorsolateral prefrontal, and medial frontal cortices in response to viewing food. In the fed state, they showed less activation in the precuneus, middle frontal, thalamic, insular, and parahippocampal cortices. After 24 weeks of LRT, while fasting, hypoleptinemic women showed greater activation in insular and inferior frontal cortices in response to viewing food. After feeding, they showed less activation in midbrain, cuneus, midcingulate, bilateral parietal, and superior prefrontal cortices. In this study, the authors showed that hypothalamic activity is modulated by LRT, which decreases functional connectivity of the hypothalamus to feeding-related areas. Despite having identified changes in brain function after LRT, this study has not shown any changes in brain structure, which the authors attribute to the fact that their patients had normal brain development during their early life leptin-sufficient period and had structurally normal brains [23]. The most relevant changes brain changes after LRT are summarized in Table 1.

7. Physiology of Leptin in the Brain

At the molecular level, leptin stimulates neurogenesis, axon growth, synaptogenesis, and dendritic morphology, both pre- and postnatal life [48]. Leptin also has neuroprotective actions, by inhibiting apoptotic cell death and improving cell survival through the regulation of apoptotic enzymes (by inhibiting the expression of Bcl-xL, caspases and TRAIL ligand, and activating the synthesis of neurotrophic factors such as BDNF), protecting against glutamatergic cytotoxicity, protecting against oxidative stress via expression of the membrane antioxidant MnSOD and stabilization of mitochondrial membranes, and promoting the proliferation of hippocampal progenitor cells [49–52]. Leptin regulates the synapse morphology of hippocampal neurons, enhancing the motility and density of dendritic filopodia [53]. Also, leptin regulates the development of oligodendroglial cells [54], which may contribute to the structural changes in gray matter. Furthermore, leptin affects neuron excitability and synaptic transmission, via the activation and trafficking of ATP-sensitive K^+ channels (in the hypothalamus) and Ca^{2+}-activated K^+ channels (in the hippocampus) [55, 56]. By enhancing NMDA receptor function, leptin facilitates the conversion of short-term potentiation into long-term potentiation, rapidly remodeling dendrites and facilitating spatial learning and memory performance in mice [55, 57–59]. Furthermore, leptin counteracts glucocorticoids' inhibitory effect on hippocampal neurogenesis [60].

Table 1: Effects of leptin replacement therapy on neural plasticity, neural function, and cognition.

	Leptin deficiency due to mutations in the leptin gene	Lipodystrophy	Acquired leptin deficiency
Brain structure	Increases in gray matter concentration in areas associated with regulation of hunger, motor control [14], relay of taste, and hippocampus [15].	Not evaluated.	No changes.
Brain function	Reduced activation of regions linked to hunger and enhanced activation of regions linked to inhibition and satiety, as well as cerebellum [16, 17]. Activation of reward- and food-processing areas [18], with sustained activation effect in the amygdala and in the orbitofrontal cortex, and decrease of the frontopolar cortex activity [19]. Activation in the anteromedial and posterolateral ventral striatum; reduced activation in the nucleus accumbens-caudate and putamen-globus pallidus regions [20]. No change in striatal D2/D3 receptor availability [21].	Increased food-related activity in the orbitofrontal cortex; decreased activity in the amygdala, hippocampus, insula, caudate, and putamen, under postprandial conditions [22].	Acute effects: Increased activation in bilateral insula, dorsolateral prefrontal, and medial frontal cortices in response to viewing food (while fasting). Less activation in the precuneus, middle frontal, thalamic, insular, and parahippocampal cortices (in the fed state). Chronic effects: Increased activation in insular and inferior frontal cortices in response to viewing food (while fasting). Decreased activation in midbrain, cuneus, midcingulate, bilateral parietal, and superior prefrontal cortices (after feeding) [23].
Cognition	Improvements of several subtests within neuropsychological functioning tests [5].	Not evaluated.	Not evaluated.

8. Leptin in Neurocognitive Disorders

Given the fact that leptin plays important roles in brain development and activity, several studies evaluated its effects on brain size and function. In healthy individuals, circulating leptin levels have been inversely correlated with total and regional brain volumes, independent of body size [45, 61, 62]. Besides further confirming the correlation between leptin levels and brain size, a prospective study of 785 healthy persons from the Framingham cohort showed that higher leptin levels were associated with a lower risk of dementia and AD in lean, leptin-sensitive people [63]. Participants in the lowest leptin quartile were at a 4-fold higher risk for developing AD in 12 years, compared to the participants in the highest quartile (25% versus 6%). The lack of association between leptin and risk of dementia was not seen in obese individuals, possibly due to leptin resistance [64]. Similar studies have also associated low leptin levels with cognitive impairment, particularly AD [65–68], but others have not identified such correlation [69, 70]. It is still unclear whether low leptin levels are involved in the pathogenesis of AD, either by directly altering neuronal and microglial physiology, by predisposing to the accumulation of toxic amyloid beta and neurofibrillary tangles [71], or by increasing central insulin resistance and inflammation [72–74]. Also, not only absolute leptin levels, but also leptin resistance may be involved in the pathogenesis of AD, as demonstrated in a study where leptin signaling was altered in the hippocampus of AD patients [75]. In transgenic mice models of AD, LRT promotes significant cognitive improvements [57]. Although leptin is a promising AD therapeutic target [76, 77], LRT clinical trials on AD patients have not been conducted so far.

9. Conclusions

More than twenty years after its discovery, leptin is now regarded as more than a regulator of food intake and energy expenditure. Animal and human models of leptin-deficiency have demonstrated that leptin is an adipokine that acts in the central nervous system and alters synaptic activity, glutamate receptor trafficking, neuronal morphology, development and survival, and microglial function. Furthermore, leptin replacement therapy in such models has elicited neuroplastic and functional changes, which translated into the activation of specific regulatory brain areas and normalization of behavioral responses to food stimuli. Interestingly, leptin replacement altered gray matter concentration of areas not related to hunger and satiety, such as the hippocampus—a brain area traditionally linked to memory and cognition. In one leptin-deficient human, treatment improved several cognitive parameters, indicating that leptin's effects in the brain may lead to clinically relevant changes. However, those changes, particularly regarding brain volume, were not evident in humans with acquired leptin-deficiency, whose brain developed normally while these patients were leptin-sufficient early in their lives. This suggests that the neuroplastic effects of leptin in leptin-sufficient individuals may not be broad enough to be detected by brain imaging, or that leptin has no neuroplastic effects at all in previously leptin-sufficient humans.

Furthermore, observational studies have shown that leptin plays a role in cognition and in the pathogenesis of Alzheimer's disease. More specifically, lower leptin levels may increase the risk for the development of Alzheimer's due to a decrease in leptin's functional and structural beneficial

effects to the central nervous system. However, results are conflicting, and confounding factors must be taken into account, such as body composition, arterial blood pressure, insulin resistance, and central inflammation.

It is unquestionable that leptin plays a role in neuroplasticity, at least in models of early-life leptin deficiency. Future studies need to establish whether a decrease in leptin's actions can lead to neurodegenerative disorders and whether the neuroplastic effects of leptin are also present in leptin-sufficient individuals. If so, clinical trials need to evaluate whether leptin replacement therapy is safe and effective for the prevention or treatment of such diseases.

Conflict of Interests

The author declares no conflict of interests.

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Research Article

History of Illicit Stimulant Use Is Not Associated with Long-Lasting Changes in Learning of Fine Motor Skills in Humans

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Little is known about the long-lasting effect of use of illicit stimulant drugs on learning of new motor skills. We hypothesised that abstinent individuals with a history of primarily methamphetamine and ecstasy use would exhibit normal learning of a visuomotor tracking task compared to controls. The study involved three groups: abstinent stimulant users ($n = 21$; 27 ± 6 yrs) and two gender-matched control groups comprising nondrug users ($n = 16$; 22 ± 4 yrs) and cannabis users ($n = 16$; 23 ± 5 yrs). Motor learning was assessed with a three-minute visuomotor tracking task. Subjects were instructed to follow a moving target on a computer screen with movement of the index finger. Metacarpophalangeal joint angle and first dorsal interosseous electromyographic activity were recorded. Pattern matching was assessed by cross-correlation of the joint angle and target traces. Distance from the target (tracking error) was also calculated. Motor learning was evident in the visuomotor task. Pattern matching improved over time (cross-correlation coefficient) and tracking error decreased. However, task performance did not differ between the groups. The results suggest that learning of a new fine visuomotor skill is unchanged in individuals with a history of illicit stimulant use.

1. Introduction

Learning of fine motor skills in humans is commonly investigated with tasks that involve visually guided movements of the hand (i.e., visuomotor tasks) [1, 2]. Tasks that involve tracking a moving target on a computer screen with movements of the hand have been particularly well characterised. Learning of such tasks is evidenced by an increase in the accuracy of pattern matching and a decrease in tracking error (i.e., deviation from the target line) over time (e.g., [3, 4]). Mechanisms that are thought to underlie learning of visuomotor tasks include changes in synaptic efficacy and functional reorganisation (plasticity) within the motor cortex (for review [5]).

Acute changes in the performance of visuomotor tasks have been observed following use of illicit drugs [6–9]. For example, the ability to track a moving target on a computer screen with movements of the hand is impaired for up to seven hours after cannabis use [6–8] (cf. [10]). Conversely, performance of a task that involves use of a joystick to keep a cursor centred in a target area (critical tracking test) is improved two hours after administration of 75 mg of 3,4-methylenedioxymethamphetamine (MDMA or "ecstasy") in adults with a history of illicit drug use [9]. However, very little is known about the acute and long-lasting effect of illicit drug use on *learning* of these tasks.

Stimulant drugs such as amphetamine, methamphetamine, and/or cocaine have the greatest potential to affect learning of fine motor skills. These drugs cause acute accumulation of primarily dopamine in the synaptic cleft (for review [11, 12]) and their use has been shown to modulate plasticity in animals [13] and in the human motor cortex [14, 15]. For example, administration of a single therapeutic dose of amphetamine enhances use- (practice-) dependant plasticity in healthy adults [14, 15] and in some stroke patients [16]. Similar findings have also been observed following

Table 1: Subject characteristics for the control, stimulant, and cannabis groups.

	Control ($n = 16$)	Stimulant ($n = 21$)	Cannabis ($n = 16$)
Age (yrs)	22 ± 4	27 ± 6*§	23 ± 5
Gender	10 M, 6 F	13 M, 8 F	10 M, 6 F
Laterality quotient	0.80 ± 0.16	0.84 ± 0.15	0.82 ± 0.12
Education (yrs)	15 ± 2	15 ± 2	16 ± 3
BDI-II score	2 ± 2	9 ± 7*	6 ± 6*
Inspection time (s)	0.73 ± 0.26	0.67 ± 0.19	0.70 ± 0.12
Lifetime alcohol use (total drinks)	55 ± 94	7,718 ± 7,236*§	2,113 ± 2,936*
Lifetime tobacco use (total cigarettes)	1 ± 2	26,943 ± 37,725*§	1,648 ± 4,490*

Data are mean ± standard deviation. *Significantly different from control group ($P < 0.05$). §Significant difference between stimulant group and cannabis group ($P < 0.05$).

administration of a single dose of levodopa [17], a drug that promotes the synthesis of dopamine.

The relationship between amphetamine and use-dependent plasticity is likely to vary in a dose-dependent manner. In rodent prefrontal cortex, injection of a low-dose of amphetamine (0.1 mg/kg) results in acute enhancement of long-term potentiation whereas high doses (10 mg/kg) abolish long-term potentiation [13]. Furthermore, high doses of amphetamine or methamphetamine, like those used illicitly, are toxic to dopaminergic neurons (for review [11, 12]). Long-lasting changes in the human motor cortex and other movement-related brain regions have also been observed in individuals with a history of illicit stimulant use [18–20]. However, it is unclear if these long-lasting pathophysiological changes alter the ability of individuals to learn new fine motor skills. Preliminary evidence suggests that motor skill learning may be unaffected in the longer term given that individuals with a history of mixed illicit stimulant use can improve their performance on the grooved pegboard test across trials and adaptation of grip force during repeated lifting of a novel object has been observed in this population [21]. Furthermore, learning of a visuomotor tracking task (pursuit rotor) was not impaired in cocaine-dependent individuals undergoing detoxification during a 21-day inpatient substance abuse treatment program [22].

The aim of the current study was to further investigate the long-lasting effect of illicit stimulant use on learning of fine motor skills. The novel aspects of the current study include (a) inclusion of a stimulant-using population with a history of primarily methamphetamine and ecstasy use, (b) inclusion of a stimulant-using population that was not undergoing detoxification, (c) inclusion of a cannabis control group to differentiate the long-lasting effects of stimulant use from cannabis use, (d) quantification of lifetime drug history for all classes of licit and illicit drugs (rather than just the drug of interest), and (e) quantification of the magnitude of pattern matching and delay between movement of the target and movement of the finger with the use of cross-correlation analysis. We hypothesised that individuals with a history of illicit stimulant use would exhibit normal performance and learning of the visuomotor tracking task compared to two control groups (nondrug using group and cannabis using group). The hypothesis was based on normal learning of the visuomotor tracking task in adults undergoing cocaine detoxification [22], a population with a high prevalence of poly-stimulant use [23].

2. Materials and Methods

Motor learning was assessed in three groups of adults. The groups were gender-matched and comprised of 22 individuals with a history of illicit stimulant use (target "stimulant" group), 17 individuals with a history of cannabis use but not illicit stimulant use (positive "cannabis" control group), and 17 individuals with no history of illicit drug use (negative "nondrug" control group). The characteristics of each group are presented in Table 1. General inclusion criteria were being aged 18–50 yrs and right hand dominant (defined as a laterality quotient of >0.4 on the Edinburgh Handedness Inventory [24]). Additional inclusion criteria for the stimulant group were use of amphetamine, methamphetamine, ecstasy, and/or cocaine on greater than 10 occasions. Additional inclusion criteria for the cannabis group were use of cannabis on greater than 10 occasions but no history of stimulant use. The cannabis group acted as a positive control group to ensure that any observed changes in motor learning were not the result of cannabis use given that cannabis use is common among stimulant users. The additional inclusion criteria for the control group was no history of illicit drug use (alcohol and tobacco use were permitted). All experimental procedures were approved by the Human Research Ethics Committee at the University of South Australia and conducted according to The Declaration of Helsinki. Written informed consent was obtained prior to participation.

2.1. Subject Screening. Subjects underwent a series of screening tests prior to the experiment. Subjects were asked to provide a urine sample for routine drug screening (PSCupA-6MBAU, US Diagnostics Inc., Huntsville, Alabama, USA) and to complete a brief medical history questionnaire [25]. Subjects were then interviewed about their lifetime and recent use of alcohol, tobacco, and illicit drugs. The interview was guided by an in-house questionnaire that listed 20 illicit drugs and requested information on other illicit drugs not listed. Items on the questionnaire included age of first use, age of regular use, duration of use, frequency of use (current and lifetime), average dose (current and lifetime),

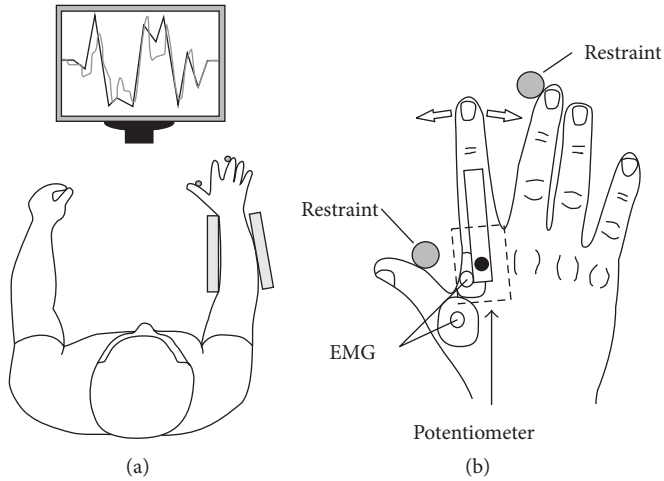

Figure 1: Experimental apparatus for the visuomotor tracking task. (a) Subjects were instructed to match their index finger metacarpophalangeal (MCP) joint angle with a moving target displayed on a computer screen. The target moved across the screen while making unpredictable upward and downward movements. Abduction of the finger moved the feedback line downward while adduction moved the feedback line upward. The maximum MCP joint angle movement was ±10° from neutral. (b) Medial-lateral movement of the index finger was recorded with a potentiometer. The axis of rotation of the potentiometer was positioned over the MCP joint. Muscle activity was also recorded from the first dorsal interosseous muscle using surface EMG.

frequency of high dose use, and time since last use defined for each drug. The number of drug overdoses and treatment for drug dependency were also noted. The final screening test involved a neuropsychological assessment of memory and cognition. Four cognitive domains were assessed. New learning was assessed with Logical Memory I and II [26], executive functioning was assessed with Verbal Trails and Verbal Fluency [27, 28], working memory was assessed with Digit Span backwards [29], and attention was assessed with Digit Span forwards [29].

Common exclusion criteria across the groups included (a) history of neurological damage and/or neurological illness prior to illicit drug use, (b) current use of prescribed medications that act on the nervous system (e.g., antidepressants), (c) frequent illicit opioid use (>5 times), and (d) positive urine test for amphetamine, methamphetamine, MDMA, cocaine, opioids, and/or benzodiazepines. Subjects who tested positive for cannabis were allowed to participate if use was >12 hours prior to the experiment. This exemption was due to the metabolite of the main active ingredient of cannabis (tetrahydrocannabinol) remaining in the body for up to 80 days after last use (for review [30]).

2.2. Experimental Protocol. The experiment began with preparation and positioning of two surface electromyographic (EMG) electrodes (Ag-AgCl, 10 mm diameter) over the muscle belly and tendon of the right first dorsal interosseus muscle. EMG activity was amplified (300 or 1000x), filtered (20–1000 Hz), and sampled at 2000 Hz using a data acquisition system (1902 with Power 1401 Interface and Signal and Spike2 software: Cambridge Electronic Design, Cambridge, UK). Subjects then completed three tasks with the right (dominant) hand.

The first task involved a three-minute visuomotor tracking task to assess motor learning. Subjects were instructed to match their index finger metacarpophalangeal (MCP) joint angle with a moving target displayed on a 22-inch computer monitor (P2210 Flat Panel Monitor, Dell Inc.). The screen was positioned two metres in front of the subject's chest and both the target and MCP joint angle were displayed (Figure 1(a)) as a solid red line on a white background. The moving target consisted of 18 unique 10 s frames (see Figures 2(a) and 2(b)) and subjects were instructed to follow the target as closely as possible. The target moved across the screen while making unpredictable upward and downward movements. Abduction of the finger moved the feedback line downward while adduction moved the feedback line upward. The maximum MCP joint angle movement was ±10° from neutral and the thumb and middle finger were restrained (Figure 1(b)). Medial-lateral movement of the index finger was recorded with a potentiometer (model 157, Vishay, NSW, Australia), with the axis of rotation positioned over the MCP joint (Figure 1(b)). The potentiometer signal was filtered (DC-100 Hz) and sampled at 2,000 Hz using the same data acquisition system.

The second task involved a brief (2-3 s) maximal isometric abduction of the index finger for normalisation of voluntary EMG recorded during the visuomotor tracking task. Three brief maximal voluntary contractions (MVCs) were performed and each contraction was separated by approximately one minute of rest to avoid fatigue. Verbal encouragement and visual feedback of force were provided. Force was recorded using a linear strain gauge (LC1205-K020, A&D Co. Ltd., Tokyo, Japan) positioned at the proximal interphalangeal joint. The thumb and middle finger were restrained. Force was recorded using the above mentioned data acquisition system. Force signals were amplified (1000x), filtered (DC-100 Hz), and sampled at 400 Hz. The EMG electrodes were removed after the last MVC.

The third task involved assessment of motor learning with the use of the Grooved Pegboard test (model 32025, Lafayette Instrument, Lafayette, IN, USA). The test involves placing 25

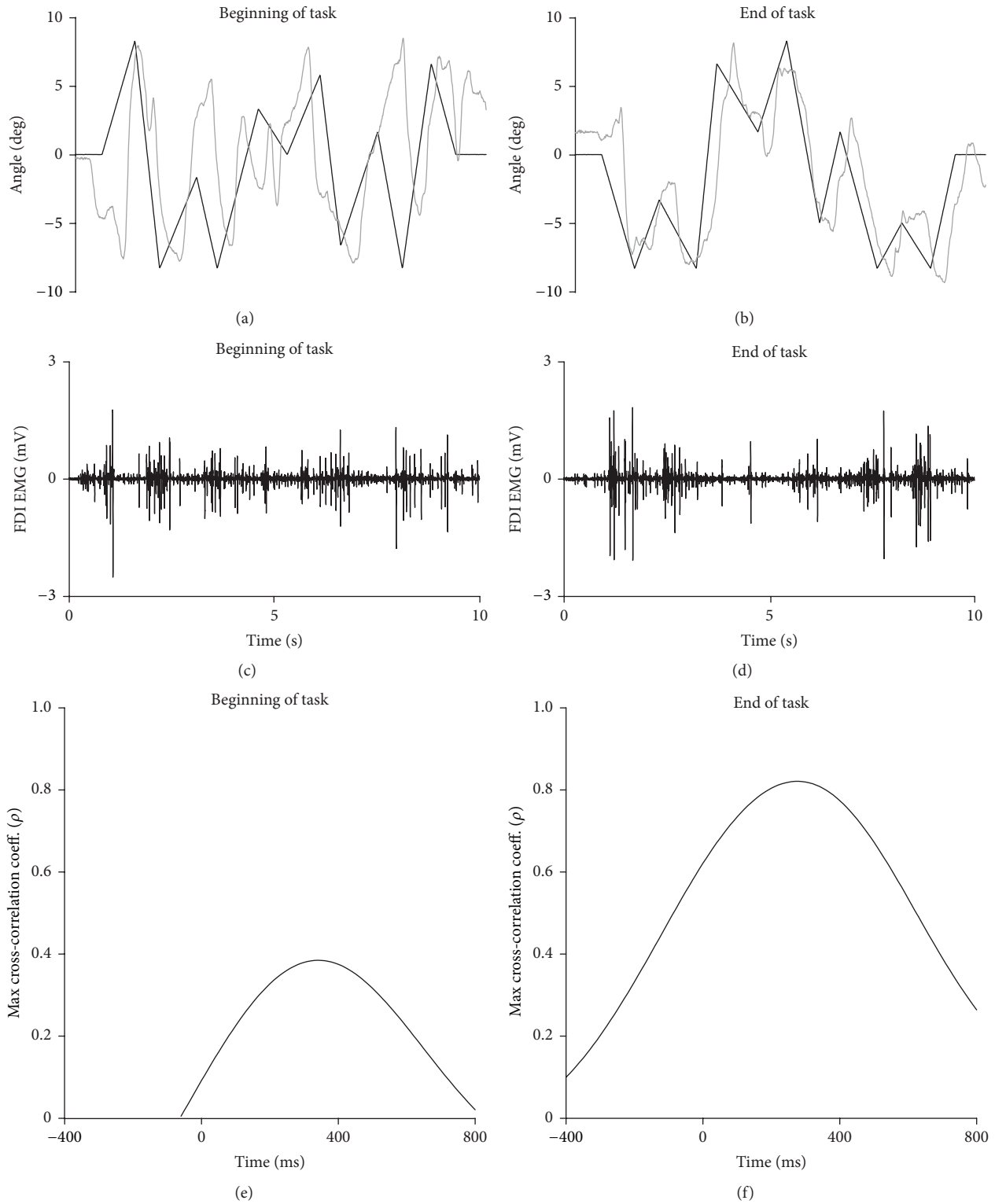

Figure 2: Single-subject data for the three-minute visuomotor tracking task. Data are from an individual in the stimulant group. (a) and (b) Raw metacarpophalangeal joint angle trace (grey line) and target line (black line) at the beginning of the first (0–30 s) and last (150–180 s) epoch, respectively. (c) and (d) Raw EMG traces for the accompanying time frame. (e) and (f) Cross-correlogram for the same subject for the first (0–30 s) and last (150–180 s) epoch, respectively.

key-shaped pegs into corresponding grooves. Subjects were instructed to complete the task (one pin at a time) as fast as possible and in a set sequence. The time taken to complete the test was recorded. Three trials were performed and each trial was separated by one minute of rest to avoid fatigue.

Factors that could alter performance of the above tasks include speed of information processing and/or symptoms of depression. These factors were assessed in the fourth and fifth tasks. Symptoms of depression (over the past two weeks) were assessed with a 21-item self-report rating scale (Beck Depression Inventory-II, [31]) and speed of information processing was assessed with the inspection time test. The inspection time test involves presentation of two parallel lines on a computer screen and indicating which of the two lines was shorter [32]. The minimum exposure time required to accurately determine the shorter line was recorded. The test is a measure of speed and efficiency of information processing independent of the motor component of reaction time.

2.3. Data Analysis. Performance on the visuomotor tracking task was assessed in 30-second epochs for each subject. The absolute difference between the target angle and MCP joint angle at each sample point was measured ("tracking error") and averaged over the 30 s epoch. The target angle was also cross-correlated with the MCP angle and the maximum cross-correlation coefficient and the lag time to achieve the maximum cross-correlation coefficient were determined (e.g., see Figures 2(e) and 2(f)). Root mean square (RMS) EMG activity was measured during the task (e.g., see Figures 2(c) and 2(d)) and expressed as a percentage of the average RMS EMG measured during the brief maximal contractions.

Group data are presented as the mean ± standard deviation (SD) in the text and mean ± standard error of the mean (SEM) in figures. Between-group comparison of subject characteristics (age, years of education), neuropsychological parameters, symptoms of depression (BDI-II score), speed of processing (inspection time), and lifetime use of alcohol (estimated total drinks) and tobacco (estimated total cigarettes) was made with one-way analysis of variance (ANOVA). Nonparametric data were transformed to ranks and a one-way ANOVA on ranks was performed (SigmaPlot for Windows Version 11.0, Systat Software Inc., San Jose, USA). Data for the visuomotor task and grooved pegboard test were analysed with two-way repeated measures ANOVA for comparison of group (between-subject factor) and time (within-subject factor). This analysis was repeated with age as a covariate. Mauchly's test of sphericity was performed and the Greenhouse-Geisser method was used to correct for nonsphericity (IBM SPSS Statistics 20, Armonk NY, USA). Post hoc discrimination between means was made with the Student-Newman Keuls procedure. Unpaired Student's t-test was used to compare lifetime cannabis use (occasions) between the stimulant and cannabis groups. Paired Student's t-test was used to compare lifetime use of ecstasy and amphetamine-like stimulants within the stimulant group. Pearson Product Moment or Spearman Rank Order correlation was used to investigate the relationship between drug-use characteristics and (a) learning (change) and (b) endpoint performance of the visuomotor task and grooved pegboard test. The relation between duration of abstinence from stimulants and the change in tracking error was also investigated with linear regression analysis (SigmaPlot for Windows Version 11.0, Systat Software Inc., San Jose, USA). Significance was set at $P < 0.05$.

Table 2: Classes of illicit drugs consumed in the stimulant and cannabis groups.

	Stimulant group	Cannabis group
Stimulants	100%	0%
Ecstasy	100%	0%
Methamphetamine	81%	0%
Cocaine	57%	0%
Pharmaceutical	14%	0%
Cannabis	100%	100%
Hallucinogens	86%	31%
Inhalants	57%	6%
Sedatives	24%	0%
Opioids	29%	0%

Data are percentage of subjects that have consumed that class of illicit drug in their lifetime. The term "hallucinogen" describes LSD (lysergic acid diethylamide), LSA (d-lysergic acid amide), "magic" mushrooms, DOI (2,5-dimethoxy-4-iodoamphetamine), *Salvia divinorum*, ketamine, and/or mescaline. The term "opioid" describes heroin, methadone, opium, and recreational use of codeine, oxycodone, and/or buprenorphine. The term "inhalant" describes amyl nitrate and/or nitrous oxide. The term "sedative" describes GHB (or "Fantasy") and/or recreational use of benzodiazepine or antidepressants.

3. Results

3.1. Subject Characteristics. One control subject, one cannabis subject, and one stimulant subject were unable to perform the visuomotor tracking task and their data was omitted from further analysis. The characteristics of the remaining 53 subjects are presented in Table 1. The groups significantly differed in age ($F_{2,50} = 4.758$, $P = 0.013$) but not in years of education, laterality quotient (index of hand dominance), or performance on the neuropsychological tests of memory and cognition. The average age of the stimulant group was 4-5 yrs older than the control and cannabis groups ($P < 0.028$) but the average age of the control and cannabis groups did not differ from one another.

3.2. Drug History. Use of alcohol and tobacco significantly differed between the groups (alcohol: $F_{2,48} = 51.043$; $P < 0.001$, tobacco: $F_{2,50} = 35.707$; $P < 0.001$). Lifetime use of alcohol (estimated total drinks) and tobacco (estimated total cigarettes) was greatest in the stimulant group and least in the control group ($P < 0.001$, Table 1).

Table 2 shows the percentage of subjects within each group that had used various classes of illicit drugs. In the stimulant group, ecstasy was the most commonly used stimulant followed by methamphetamine, cocaine, and recreational use of pharmaceutical stimulants. Polydrug use was common in the stimulant group and less common in the cannabis group. All subjects in the stimulant group had used cannabis and the majority of subjects had used hallucinogens

Table 3: Summary of lifetime use of stimulants and cannabis in the stimulant group.

Subject	Total stimulants	Amphetamines	Ecstasy	Cannabis
1	2,241	2,072	169	28
2	833	832	1	13
3	828	402	426	3,675
4	367	211	156	4,380
5	332	228	104	1,251
6	213	3	210	120
7	209	208	1	6,570
8	199	93	106	23
9	156	3	153	1,529
10	57	5	52	4,380
11	38	26	12	5,616
12	36	10	26	474
13	31	3	28	876
14	27	26	1	270
15	22	2	20	1,456
16	19	8	11	6
17	19	1	18	15
18	18	—	18	153
19	17	4	13	2,763
20	16	10	6	1,092
21	12	3	9	72
Mean	271 ± 513	207 ± 483	73 ± 104	1,655 ± 2,061

Single-subject and mean data are presented (number of times used). The term "amphetamine" describes amphetamine and amphetamine-like drugs such as methamphetamine, cocaine, dexamphetamine, Ritalin, and pemoline (one subject). The term "ecstasy" describes ecstasy, MDA (3,4-methylenedioxyamphetamine, one subject), and MCAT (mephedrone, one subject).

(primarily lysergic acid diethylamide or "LSD" and "magic" mushrooms) and inhalants (primarily nitrous oxide). Illicit use of sedatives and opioids was uncommon and total lifetime use of these drugs was low in the stimulant group (sedatives: 15 ± 30 occasions; opioids: 3 ± 1 occasions).

Table 3 shows single subject and group data for lifetime use of amphetamine-like stimulants, ecstasy, and cannabis in the stimulant group. Lifetime use of amphetamine-like stimulants was greater than lifetime use of ecstasy. The average duration of abstinence from stimulants was 1.6 ± 3.0 yrs (range: 7 days–12 yrs). Lifetime use of cannabis was significantly greater in the stimulant group (1,655 ± 2,061 occasions) than in the cannabis group (222 ± 334 occasions; $P = 0.009$) and the average duration of abstinence from cannabis was 224 ± 635 days (range: 1 day–8 yrs) and 395 ± 617 days (range: 1 day–5 yrs) for each group, respectively. No drug overdoses were reported in the control and cannabis groups, but five subjects in the stimulant group reported having experienced a drug overdose.

3.3. Visuomotor Tracking. Figures 2(a) and 2(b) show raw traces of MCP joint angle from a single subject in the stimulant group at the beginning and end of the visuomotor task. At the beginning of the task (first epoch: 0–30 s), the MCP joint angle trace partially resembled the target pattern (maximal cross-correlation coefficient: 0.39, lag time: 340 ms, tracking error: 4.4°; Figure 2(e)). Performance improved over time evidenced by a greater maximal cross-correlation coefficient (0.82) and reduced tracking error (2.7°) and lag time (280 ms) in the final epoch (150–180 s; Figure 2(f)).

Figure 3 shows group data for the visuomotor tracking task. There was a significant main effect of time on the absolute difference between the target angle and MCP angle (i.e., tracking error, $F_{5,250} = 30.687$, $P < 0.001$; Figure 3(a)). Tracking error significantly decreased over the first minute of the task (from epoch 1 to epoch 2, $P < 0.044$) but remained unchanged thereafter. However, tracking error did not differ between the groups and there was no significant group-by-time interaction.

The accuracy of pattern matching between the target angle and MCP joint angle was assessed for each subject by cross-correlation of the target angle with the MCP joint angle. A cross-correlation coefficient of 1 would indicate a perfect match whereas a cross-correlation coefficient of 0 would indicate no match. Across the groups, there was a significant main effect of time on the maximum cross-correlation coefficient ($F_{5,250} = 43.770$, $P < 0.001$, Figure 3(b)). The maximum cross-correlation coefficient did not significantly differ between groups, but there was a significant group-by-time interaction ($F_{10,250} = 2.684$, $P = 0.010$). The interaction arose from a subtle difference in the timing of improvement and attainment of a plateau in performance between groups, but not in the magnitude of improvement. In the stimulant group, the maximum cross-correlation increased over the first minute of the task (from epoch 1 to epoch 2) but remained relatively unchanged thereafter ($P < 0.001$, except for the epoch 2 versus epoch 4 comparison: $P = 0.029$). In the control and cannabis groups, the maximum cross-correlation coefficient increased for longer, up until 2–2.5 mins into the task (from epoch 1 to epochs 4-5, $P < 0.039$).

Voluntary RMS EMG (% of maximum) did not significantly differ over time or between groups (average across epochs: control = 11.0 ± 6.7%, stimulant = 10.7 ± 3.8%, and cannabis = 14.4 ± 10.8%; data not shown). The lag time between movement of the target and movement of the MCP joint angle also did not significantly differ over time or between groups (average across epochs: control = 302 ± 298 ms, stimulant = 423 ± 232 ms, and cannabis = 351 ± 221 ms; data not shown). There was also no significant group-by-time interaction on voluntary RMS EMG or lag time.

The analysis of each parameter in the visuomotor tracking task was repeated with age as a covariate. No significant main effect of group was observed after accounting for age.

In the stimulant group, the relation between stimulant drug use characteristics (total lifetime use or duration of abstinence) and learning and endpoint performance (epoch 6) on the visuomotor tracking task was explored with correlation analyses. No significant correlations were observed. However, there was a trend for a correlation between duration of abstinence from stimulants and change (reduction) in tracking error across the visuomotor task ($r = 0.383$, $P = 0.085$). Individuals with a shorter duration of abstinence

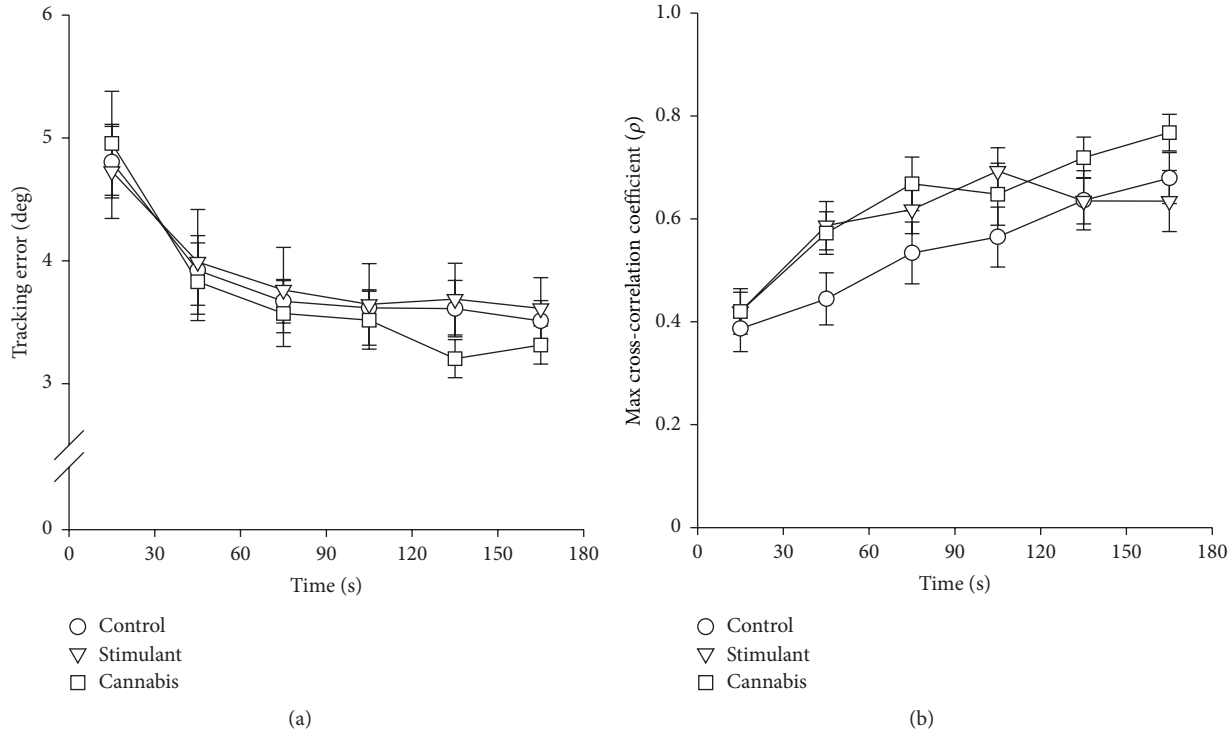

Figure 3: Group data showing performance during the visuomotor tracking task. (a) Tracking error. (b) Maximum cross-correlation coefficient (derived from cross-correlation of the target angle with metacarpophalangeal joint angle). Data for the control (circles), stimulant (triangles), and cannabis (squares) groups are shown.

tended to exhibit less change in tracking error (i.e., less improvement) than individuals with a longer duration of abstinence (Figure 4). In the cannabis group, there was no significant correlation between cannabis drug use characteristics and learning and endpoint performance of the visuomotor task.

3.4. Grooved Pegboard. Figure 5 shows group data for the grooved pegboard test. There was a significant main effect of trial on the grooved pegboard test ($F_{2,100} = 104.837$, $P < 0.001$). Performance time significantly decreased across trials, indicating an improvement in performance. There was no significant main effect of group on the grooved pegboard test, but there was a significant group-by-trial interaction ($F_{4,100} = 4.391$, $P = 0.003$). Performance improvement was evident across all three trials in the control and stimulant groups ($P < 0.008$), but improvement was only observed between trials one and two in the cannabis group ($P < 0.001$). There was no significant correlation between stimulant and cannabis drug use characteristics (total lifetime use or duration of abstinence) and learning and endpoint performance (trial three) on the grooved pegboard test in the stimulant and cannabis groups, respectively. Analysis of the grooved pegboard data was repeated with age as a covariate. No significant main effect of group was observed after accounting for age.

3.5. Symptoms of Depression and Speed of Information Processing. Symptoms of depression (BDI-II score) significantly differed between the groups ($F_{2,50} = 7.352$, $P = 0.002$). As expected, subjects in the stimulant group ($P = 0.001$) and cannabis group ($P = 0.026$) experienced significantly more symptoms of depression than subjects in the nondrug using control group (Table 1). Three subjects in the stimulant group and one subject in the cannabis group had received a formal diagnosis of depression (after commencing illicit drug use), but none were being medicated at the time of the experiment. The groups did not significantly differ in speed of information processing (inspection time, Table 1).

4. Discussion

Performance of the visuomotor tracking task and grooved pegboard test improved over time. The improvement in performance is indicative of motor learning. We have shown for the first time that individuals with a history of illicit stimulant use exhibit (a) normal performance of a visuomotor tracking task and (b) normal learning of fine motor skills. The latter finding supports our initial hypothesis.

Performance of the visuomotor tracking task requires an awareness of where the index finger is in space (proprioception) and relative to the target as well as integration of visual input and motor output. Sensory feedback from the periphery and ongoing modulation of movement are also important. The lack of a between-group difference in performance of the visuomotor tracking task suggests that use of illicit stimulants drugs is not associated with long-lasting changes in the physiology that underlies performance of this task. Alternatively, movement-related brain regions

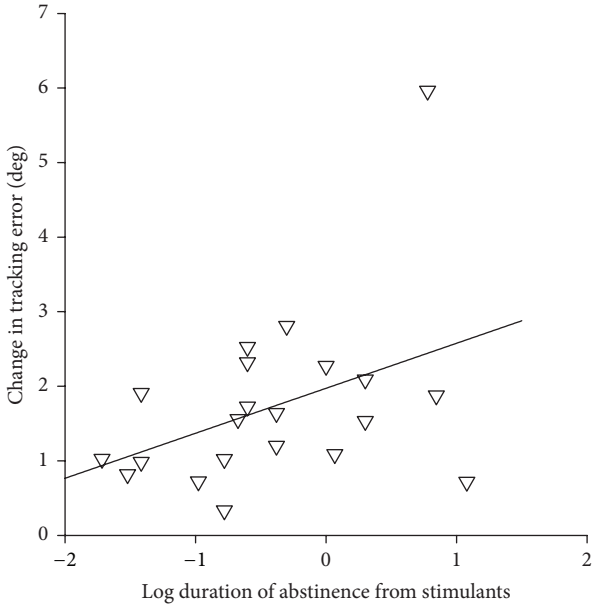

Figure 4: Correlation between duration of abstinence from stimulants and change in the tracking error across the visuomotor task. Single subject data for the stimulant group are shown and log duration of abstinence is plotted on the x-axis. The correlation did not reach statistical significance ($r = 0.383$, $P = 0.085$). Solid line shows the result of a linear regression analysis ($P = 0.070$).

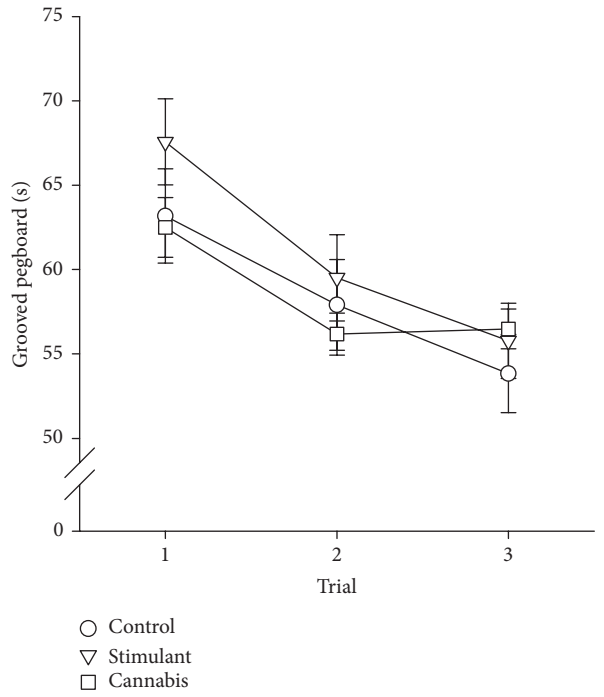

Figure 5: Group data showing performance during the grooved pegboard test. Data for the control (circles), stimulant (triangles), and cannabis (squares) groups are shown.

may employ compensatory mechanisms that are capable of overcoming any drug-related deficits.

Learning of the visuomotor tracking task occurred across groups. Learning was evidenced by an increase in the accuracy of pattern matching (i.e., increase in the cross-correlation coefficient) and a decrease in the tracking error (i.e., difference between the target and MCP joint angle) over time. The majority of the improvement was observed in the first 90 s of the task with a plateau in performance observed thereafter. The improvement in performance did not appear to be associated with changes in first dorsal interosseous muscle activity or the lag between movement of the target and movement of the MCP joint because these parameters did not significantly change over time.

Learning of the visuomotor tracking task did not differ between groups. This suggests that learning of visuomotor tasks that involve fine movements of the hand is unaffected in individuals with a history of illicit stimulant use. Lifetime use of illicit stimulants was high in the stimulant group (271 ± 513 occasions) so the existence of any long-lasting effects of illicit stimulant use on learning should have been apparent in this group. However, subjects in the stimulant group had been abstinent from stimulants for an average of 1.6 ± 3.0 yrs (range: 7 days–12 yrs) and any deficits in learning and/or task performance could have recovered during the weeks or months following cessation of use. The trend for a positive correlation between duration of abstinence from stimulants and change in tracking error supports this view ($P = 0.085$, Figure 4). However, a larger sample size would be required to confirm this.

The sensitivity of the equipment and data analysis was sufficient to observe a between-group difference if one had been present. The methodology enabled detection of a very small improvement in tracking error, of as little as one degree between the joint angle and target angle, during the first minute of the visuomotor tracking task and this small improvement was statistically significant. Thus, detection of a between-group difference of as little as one degree would have been measurable if it had been present but this was not the case.

The current study is the first to demonstrate normal performance and learning of a visuomotor tracking task in individuals with a history of illicit stimulant use. Normal performance of another fine motor skill, the grooved pegboard test, has been previously reported in individuals with a history of mixed stimulant use and confirmed in the current study [33–36]. However, there have also been reports of slower performance time on the grooved pegboard test in this population [20, 37–41], but the impairment became less apparent with increasing duration of drug abstinence [38]. Cannabis users who have abstained from cannabis use for a long period of time (52 ± 17 months) also exhibit normal performance of the grooved pegboard test [42]. We have added to the literature on this topic by investigating both performance and learning of the grooved pegboard task. The time to complete the grooved pegboard test decreased across trials, suggesting an improvement in performance and thus learning of the task. A significant group-by-trial interaction was also observed. Performance improvement was evident across all three trials in the control and stimulant groups, but

improvement was only observed between trials one and two in the cannabis group.

The lack of an association between history of illicit stimulant use and performance and learning of fine motor skills is surprising given that long-lasting changes in movement-related brain regions have been observed in this population. Studies involving noninvasive brain stimulation show that history of predominantly methamphetamine and ecstasy use is associated with a long-lasting increase in excitability of the motor cortex and corticospinal pathway [18]. Changes in excitability of this pathway have also been observed in abstinent cocaine-dependent individuals [43, 44]. Neuroimaging studies also show reduced dopamine reuptake transporter [45] and dopamine (D2) receptor availability in the striatum of abstinent methamphetamine users [46]. Furthermore, abnormal morphology of the substantia nigra, a brain region with a high density of dopaminergic neurons, has also been observed in individuals with a history of primarily methamphetamine and ecstasy use [19]. The latter abnormality is a strong risk factor for developing Parkinson's disease later in life [47].

Factors other than illicit stimulant use have the potential to affect learning and performance of fine motor skills. Neuropsychological factors can influence learning and performance but are unlikely to have affected the results of the current study. No between-group differences were observed in the score on the inspection time test (speed of information processing) or tests of memory and cognition. Group data for these tests were also above (Logical Memory I and II, Verbal Trails, and Digit Span) or within one standard deviation (Verbal Fluency) of published normative data for the general population [48–51]. Attention and symptoms of depression are also unlikely to have influenced the results of the current study. The duration of each motor task was short (1–3 mins) and performance on the Digit Span forwards test (attention) did not differ between groups. The drug-using groups exhibited more symptoms of depression than the nondrug using group, but the groups did not differ in learning or performance of the tasks.

Other factors that could influence learning and performance of fine motor skills include age, gender, and lifetime use of alcohol, tobacco, and/or cannabis. Gender and cannabis use were accounted for in the experimental design. However, the possible effects of age and lifetime use of alcohol and tobacco were not accounted for because these parameters were not adequately matched between groups. The age of subjects in the stimulant group (27 ± 6 yrs) was on average 4-5 yrs older than subjects in the positive (cannabis: 23 ± 5 yrs) and negative (nondrug: 22 ± 4 yrs) control groups. The small age difference between stimulant and cannabis users is to be expected based on published epidemiological data on drug use patterns. In Australia, the prevalence of cannabis and stimulant use is highest in adults aged 20–29 yrs (21.3% and 5.9–9.9%, resp.), but the onset of cannabis use tends to occur at an earlier age than the onset of stimulant use (prevalence in individuals aged 14–19 yrs is 21.5% and 4.7–6.2%, resp.) [52]. The mean age of the groups matched the epidemiological data implying that the study sample was representative of the wider drug-using population. The small age difference between the groups is unlikely to have affected the results of the current study because no between-group differences in performance or learning were observed and the magnitude of learning associated with a comparable visuomotor task is similar for young (aged 20–35 yrs) and older (aged 60–75 yrs) adults [3]. The greater lifetime use of alcohol and tobacco in the drug using groups is also to be expected based on published epidemiological data. Individuals who use stimulants and/or cannabis are well known to consume more alcohol and tobacco than individuals with no history of illicit drug use [52, 53]. The lack of a between group difference in performance and learning of fine motor skills in the current study suggests that the greater lifetime use of alcohol and tobacco had a minimal effect on the outcome of the study.

In summary, the results of the current study suggest that history of illicit stimulant use is not associated with long-lasting changes in the learning and performance of fine visuomotor skills. The results of the current study have implications for rehabilitation of movement deficits in this population. Individuals with a history of illicit stimulant use are capable of learning new fine motor skills if this were required in a rehabilitation program. The results of the current study also have implications for the potential use of amphetamine as a therapeutic aid for stroke rehabilitation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Deep Brain Stimulation for Obesity: From a Theoretical Framework to Practical Application

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Obesity remains a pervasive global health problem. While there are a number of nonsurgical and surgical options for treatment, the incidence of obesity continues to increase at an alarming rate. The inability to curtail the growing rise of the obesity epidemic may be related to a combination of increased food availability and palatability. Research into feeding behavior has yielded a number of insights into the homeostatic and reward mechanisms that govern feeding. However, there remains a gap between laboratory investigations of feeding physiology in animals and translation into meaningful treatment options for humans. In addition, laboratory investigation may not be able to recapitulate all aspects of human food consumption. In a landmark pilot study of deep brain stimulation (DBS) of the lateral hypothalamic area for obesity, we found that there was an increase in resting metabolic rate as well as a decreased urge to eat. In this review, the authors will review some of the work relating to feeding physiology and research surrounding two nodes involved in feeding homeostasis, nucleus accumbens (NAc) and hypothalamus, and use this to provide a framework for future investigations of DBS as a viable therapeutic modality for obesity.

1. Introduction

Obesity is a pervasive global health problem [1, 2]. The World Health Organization (WHO) estimates that nearly 500 million people worldwide are obese [3]. A plethora of weight loss solutions exist, including gastric bypass surgery, but none have emerged as a durable solution. Surgical options for obesity include gastric bypass and banding surgery, which attempt to modulate the physiology of the gastrointestinal (GI) system to produce weight loss [4, 5]. However, as evidence indicates, much of the initial weight loss is regained and long-term complications [6, 7] from manipulation of the GI tract have made it less attractive in recent years. Scientific inquiry in recent years has found that obesity involves a complex interplay of neural networks that contribute to feeding behavior. While some argue that obesity is simply an imbalance between energy expenditure and food intake, evidence continues to mount that obesity may be the result of preserved feeding patterns that evolved in our ancestors due to inconsistent food availability. These behavioral patterns are particularly vulnerable to the ready availability of food and increased food palatability, both of which contribute to the growing epidemic of obesity. The failure of surgical modification of the GI tract to "cure" the epidemic of obesity may be a result of not addressing the underlying neurophysiologic basis of the disease. Feeding behavior then may be an interplay between a physiologic need for food and the reward system that powerfully motivates excessive eating in some individuals [8, 9].

Deep brain stimulation (DBS) has emerged as a minimally invasive, reversible method of neuromodulation first approved for movement disorders with expanded applications to a spectrum of neuropsychiatric disease including major depressive disorder (MDD) [10, 11], obsessive-compulsive disorder (OCD) [12–14], Tourette's syndrome (TS) [15–17], and addiction [18]. Insights gained from these studies, coupled with abundant animal research, led us to conduct the first human trial of DBS of the lateral hypothalamic area (LHA) for refractory morbid obesity [19]. In this paper, the authors will review some of the work relating to feeding physiology and research surrounding two nodes involved in feeding homeostasis, nucleus accumbens (NAc)

and hypothalamus, and use this to provide a framework for future investigations of DBS as a viable therapeutic modality for obesity.

2. Physiologic Regulation of Feeding

Short- (cholecystokinin (CCK) and ghrelin) [20–22] and long-term (leptin, insulin) [20–22] satiety signals physiologically signal a nutrient abundance or deficit and regulate feeding behavior. Gastric distention by food intake causes the release of CCK, which acts on the nucleus of the solitary tract (NTS), which integrates taste and satiety information. Ghrelin release, in contrast, from the stomach peaks shortly before meal initiation and levels are found to rise after weight loss and may contribute to weight regain [23]. The discovery of leptin as a circulating satiety signal led to investigation in several human trials but disappointing results and the discovery that leptin resistance is common amongst obese individuals curtailed enthusiasm for its use [24]. Leptin is a small peptide that traverses the blood-brain barrier and acts on the arcuate nucleus of the hypothalamus, LHA, and the NTS. Though elevated leptin is a physiologic marker for adequate long-term energy stores, it also regulates feeding behavior during meals by augmenting the satiety response to CCK [25, 26].

Lesioning studies, first by Hetherington and Ranson [27] and later by Anand and Brobeck [28], paved way to the "classic" teaching of hypothalamic control of feeding behavior by two competing systems, one in the LHA and the other in the ventromedial hypothalamus (VMH). Lesions of the LHA resulted in cessation of feeding behavior and severe anorexia, while those of the VMH resulted in hyperphagia and obesity. Scientific inquiry in the decades following these landmark studies has revealed that feeding physiology cannot be distilled into a simple binary system of "on" and "off." Instead, there appear to be complex interactions between clusters of hypothalamic nuclei that are powerfully governed by long-term hormonal signals such as leptin and insulin [29, 30]. Both hormones act on the arcuate nucleus of the hypothalamus, located inferolaterally to the walls of the third ventricle as well as the lateral hypothalamic area.

The arcuate nucleus contains two distinct subpopulations of neurons: those expressing neuropeptide Y (NPY) and agouti-related peptide (AGRP) as well as those expressing proopiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) [31, 32]. The LHA contains neurons that produce melanin concentrating hormone (MCH) [33, 34] and orexins [35, 36]. Feeding behavior is promoted by NPY/AGRP and MCH/orexin neurons in the arcuate and LHA nuclei, respectively, while satiety is mediated by POMC/CART neurons [20, 37]. The physiological effects of melanocortin peptides are mediated by binding to melanocortin receptors, of which the melanocortin-3 and melanocortin-4 (MC3R and MC4R) subtypes are highly expressed in the central nervous system. In contrast to the agonist activity of melanocortins on the MC3 and MC4 receptors, NPY and AGRP are antagonists for these same receptor subtypes and thereby exert opposing physiological effects. In addition, NPY neurons have synaptic contacts on POMC neurons and have a net inhibitory effect and thus promote feeding [30, 31]. Obesity in mouse models can be generated by deletions or mutations of either the POMC gene [38] or MC3R/MC4R [39, 40] genes, and a similar phenotype is seen in humans that have deficiency of the MC4R [41, 42]. This evidence substantiates the importance of the melanocortin peptide and its associated receptors on energy homeostasis. Furthermore, leptin receptors are found on both POMC and NPY neurons, with the net effect of inhibiting NPY neurons and activating POMC neurons, thus resulting in satiety [30, 31]. In support of leptin's modulatory role on these distinct neuronal populations are mouse models that show that the obesity syndrome classically studied in leptin-deficient mice ($Lep_{ob/ob}$), when crossed with NPY-null mice, reduces obesity as compared with $Lep_{ob/ob}$ mice alone [43]. In a physiologically normal system, the fall in circulating leptin following weight loss decreases its inhibitory effect on the NPY/AGRP and LHA neurons and promotes feeding behavior (Figure 1) [20, 37]. In leptin-deficient mice, the downstream targets (NPY/AGRP neurons) are therefore constitutively active promoting hyperphagia and resulting in obesity. In contrast, VMH lesions tend to destroy the downstream targets of leptin action and leave the actions of the LHA unopposed, generating obesity.

2.1. Human Studies of DBS in the Hypothalamus. Evidence from animal models and lesioning studies led to two studies in which the VMH was targeted in 1 patient for obesity [44] with no effect on weight loss, though vivid autobiographical memories were enhanced, presumably by forniceal activation. Confirming the untoward effects of VMH stimulation, Wilent et al. exemplified the adverse psychogenic manifestations associated with this region when panic-attacks were induced in a graded manner with electrical stimulation of the VMH [45]. These unwanted adverse effects have since waned interest in targeting the VMH for obesity.

In our FDA-approved pilot study of 3 patients with refractory morbid obesity (all of whom had failed gastric bypass), we were able to demonstrate increases in resting metabolic rate in 2 of 3 patients using monopolar stimulation. Interestingly, traditional programming parameters as used for DBS in movement disorders did not result in changes in metabolism [19]. Biochemical profiles of hormones involved in obesity including T4, T3, insulin growth factor (IGF), leptin, AGRP, ghrelin, and NPY were all measured at baseline and after LHA-DBS with no change following stimulation. The finding that there was no change in these hormonal markers implies two possibilities: the fact that DBS may work as a modulator independent of hormonal changes or the fact that long-term follow-up is needed to determine whether changes do occur. Long-term programming at the RMR-optimized settings resulted in a decreased urge to eat as well as increased subjective feelings of energy that resolved when the stimulator was turned off, even in a blinded fashion. These findings suggest that DBS for obesity may involve distinct neural networks that need further investigation to determine optimal stimulation settings. Neuropsychological scales were also administered to all 3 patients that measured binge eating, body image, and feelings of hunger. Binge eating was reduced

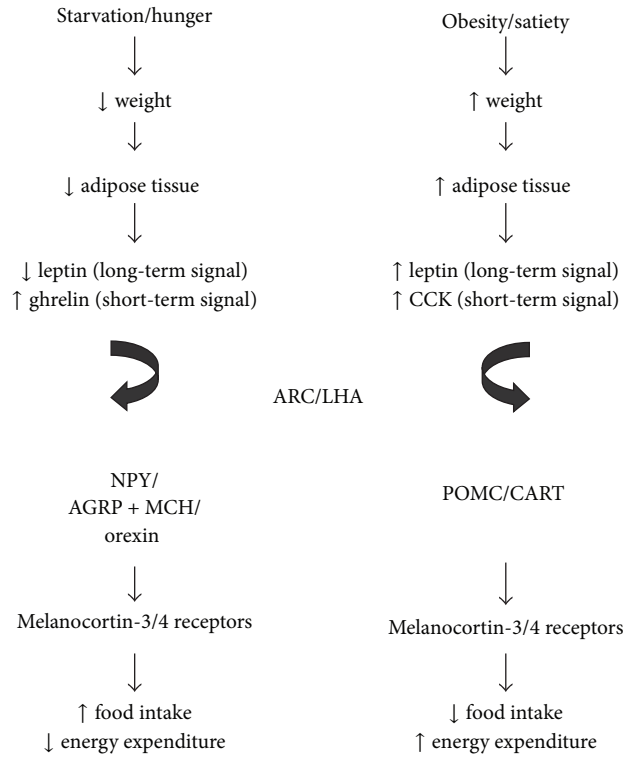

Figure 1: Starvation and satiety have opposing effects on levels of long-term (leptin, insulin) and short-term (ghrelin, CCK) signals that act on distinct neuronal populations within the hypothalamus. The effects of these neurons are mediated by melanocortin receptors and ultimately lead to increased or decreased intake based on the organism's needs.

in one patient, and importantly this same patient reported reduced feelings of hunger. The two remaining patients while not showing any changes in binge eating behavior or feelings of hunger did show improvements regarding body image. While the weight lost by our patients was not significant, our pilot study was able to confirm the safety of LHA stimulation with few adverse events, laying groundwork for future studies.

3. Reward Integration of Feeding

Our work, in addition to those of others, has shown that combating obesity is more complex than simply changing feeding behavior but may also require modulation of reward networks. Leptin and insulin, in addition to modulating hypothalamic regions, have also been found to exert influences on reward circuitry such as the ventral tegmental area (VTA) [37, 46–48]. The VTA, located in the midbrain, provides much dopaminergic input to the nucleus accumbens (NAc), striatum, and other brain areas and is known to have receptors for leptin and insulin. As with hypothalamic areas, leptin and insulin appear to tonically inhibit the VTA, as demonstrated by experiments in which centrally administered leptin decreases sucrose preference [47], and it also decreases firing of neurons in the VTA [48]. In addition, the reward value of drugs has been shown to increase in states of food deprivation, confirming leptin's inhibitory role in the reward system [49–51]. Leptin circulates in proportion to body fat mass, and levels fall in states of food deprivation. This drop in leptin (see above) may release feeding centers (ARC/LHA) and the VTA from tonic inhibition and coupled with a compensatory rise in ghrelin may prime feeding behavior to restore lost weight.

3.1. Dopamine's Role in Reward. Examining the role of dopamine neurotransmission is essential for understanding reward integration of feeding behavior. Some researchers have also used neuroimaging studies to suggest that reward hypofunction is central to the pathophysiology of obesity as demonstrated by decreased D2 receptor availability in obese individuals [9]. Proponents of this hypothesis suggest that obesity may stem from a compensatory drive by individuals to "restore" normal levels of pleasure by overeating [52, 53]. However, abundant animal research into dopamine's function in the last two decades has revealed that it may not necessarily be responsible for the hedonic impact of stimuli and that there may exist a distinct but important dissociation between "liking" and "wanting" [8, 9, 54]. The dissociation of "liking" (hedonic experience) and "wanting" (desiring a stimulus) may be important in obese individuals, who may overeat because of a *desire* or *craving* that is out of conscious control than enjoying the hedonic component of eating. Obesity may therefore have similar pathophysiology to drug addiction, a view endorsed by some.

Dopamine has long been touted as the "pleasure" neurotransmitter [55] and that it is necessary for subjective feelings of hedonia. Animal experiments have shown that pharmacologic silencing (using 6-OHDA) of up to 99% of DA

in the NAc and striatum failed to decrease "liking" responses to sweet rewards [54, 56]. Furthermore, experiments in mice where extracellular DA was artificially increased through knockdown of the dopamine transporter (DAT) failed to increase "liking," though "wanting" (food-seeking behavior) was increased [57, 58]. This evidence suggests that dopamine may not be necessary to mediate feelings of reward [8, 9] in sharp contrast to other experiments that have shown that opioid, cannabinoid, and benzodiazepine administration demonstrate increased "liking" reactions to sweet reward [59–64]. The dissociation between hedonia and DA neurotransmission is also evident in patients with Parkinson's disease (PD), who have subjective hedonic experiences similar to control patients [65]. Other work has also shown that diet induced DA deficiency in healthy human subjects did not change subjective feelings of "liking" a cocaine reward though desire for cocaine seemed to wane [66]. Dopamine dysregulation in obese individuals may therefore be a consequence of the disease process and may not play a causal role in the development of obesity.

4. Hedonic Hotspots

The hedonic experience of food activates a number of brain structures that have reciprocal connections with each other. These structures include neocortical structures such as the orbitofrontal, anterior cingulate, and insular cortices, as well as phylogenetically older subcortical areas such as the NAc, ventral pallidum, amygdala, and parabrachial nucleus of the pons. Particularly compelling is that within these structures lie hedonic "hotspots" which amplify both desire (wanting) and hedonia (liking) for food. This evidence suggests that any modulation of feeding behavior (as in our study with the LHA) may not be as effective because it does not alter the *desire* for food. Two decades of animal research [8, 9, 54, 58, 59] has revealed that behavioral homologues exist between animal, primate, and human models where "liking" reactions are distinct from "disliking" or aversive reactions.

4.1. Nucleus Accumbens. The significant role of the nucleus accumbens in reward processing is undisputed. The NAc has been targeted in both animal and human studies, with the latter focusing on neuropsychiatric conditions such as MDD, OCD, and Tourette's as well as addiction [10–18]. In recent years, significant advances have shown that the NAc is made of two distinct regions: the core and the shell that subserve specific functions [67–69]. The shell of the accumbens receives afferents from the ventral medial prefrontal cortex as well as the VTA, areas that have been implicated in drug-seeking behavior [70, 71], while the core is innervated predominantly by the substantia nigra, involved with motor planning and execution [72]. These differences in functionality between the core and shell are recapitulated by their efferent projections, with the former projecting to premotor and supplementary motor cortices and the latter to subcortical motor areas, the LHA, and amygdala [69, 73, 74]. In rodent models, it was found that a small area in the rostrodorsal medial shell of the NAc by injection of opioid or endocannabinoid agonist significantly increased both "liking" (orofacial reactions) and wanting for food rewards [63, 64]. Surprising, however, was that opioid or endocannabinoid agonists into areas outside this "hotspot" increased wanting for food without changing the absolute number of liking orofacial reactions. This finding suggests that the dissociation between "liking" and "wanting" may be applicable to humans. In parallel to drug addicts, obese individuals may simply overeat not because they "like" food more, but simply "crave" it more than normal individuals when exposed to palatable food or food-related cues. It also highlights the importance of any future study targeting the NAc for obesity, as the goal is to modulate craving without changing the hedonic experience of food.

4.2. Ventral Pallidum. A hedonic hotspot has also been identified in a small area in the posterior part of the ventral pallidum and recent studies have shown that it is also intricately involved in the processing of food reward. Historical lesioning studies of the 1960s and 70s damaged the LHA, resulting in hypophagia, but also decreased "liking" reactions to sweet reward by damaging the ventral pallidum [8, 9, 75, 76]. Furthermore, as with the previously mentioned NAc hotspot, an opioid agonist injected increased the number of "liking" orofacial reactions while neuronal cell death or chemical inactivation (by GABA agonists) led to aversive reactions even for normally palatable sweet rewards [8, 9, 77]. It has also been found that pallidal neurons code for reward based on physiologic states. In normal conditions, pallidal neurons fire vigorously when sweet reward (sucrose) is administered but not to excessively salty water in rodents. However, the induction of salt appetite by administration of diuretics causes pallidal neurons to fire in a similar fashion to baseline firing for sweet reward [78, 79]. This is compelling evidence that the physiologic state of an organism is crucial in perception of whether a stimulus is perceived as pleasurable. Importantly, there also appear to be direct projections of orexin neurons [35, 36] to the pallidal hotspot, coupling feeding behavior directly with hedonic experience [80, 81]. Orexigenic neurons through direct projections to posterior ventral pallidum may provide incentive to seek food beyond physiologic need by acting on one node in the reward system. While evidence of these hotspots is compelling, it remains to be determined whether such homologous regions exist in humans. Research has also shown that in addition to modulating "liking" independently, the NAc and pallidum work together [82]. Injection of an opioid agonist into either NAc or pallidum activates the other structure and this dual activation may act synergistically to produce "liking."

5. The Incentive Salience Theory

Developed by Berridge and Robinson et al. [8, 9], the incentive salience theory is an attempt to elucidate the true role of dopamine neurotransmission. At its essence, incentive salience is distinctly different from the hedonic experience of a reward such as food. According to Berridge and Robinson, incentive salience is the "active assignment of salience and attractiveness to visual, auditory, tactile, or olfactory stimuli that are themselves intrinsically neutral. Salience attribution

possesses the qualities of wanting and desiring, but these need to be distinguished from the experience of sensory pleasure." [8] Incentive salience then is the mechanism by which "wanting" a particular reward stimulus (food, sex, drugs, etc.) is generated. This is distinctly different from explicit or declarative "wanting" which is mediated at higher cortical levels [8, 9]. Most importantly, incentive salience is dynamic and highly dependent on the physiologic state of the organism and drives behavior subconsciously.

Central to the generation of incentive salience of a particular reward stimulus is mesolimbic dopaminergic neurotransmission. Particularly, palatable food may take advantage of normal neural mechanisms that evolved to reinforce feeding behavior when food was scarce to allow an organism to survive. The importance of incentive salience to understanding the pathophysiology of obesity is that it provides a link between the physiologic *need* to eat and the hedonic aspects of food consumption.

Berridge's three-stage model of incentive salience [8] is based on early Pavlovian experiments involving classical conditioning. In the first stage, a new stimulus (such as palatable food) is encountered by an organism and is intrinsically neutral. If the stimulus (i.e., taste) is perceived as pleasurable, it activates "liking" and secondarily activates "wanting." The rewarding stimulus that predicted the hedonic experience is assigned incentive salience. Upon exposure to the rewarding stimulus subsequently (food), the incentive salience of whatever predicted it (contextual environment such the location, aroma of food, etc.) is potentiated. In the final stage, mesolimbic dopaminergic transmission occurs to generate incentive salience anew each time the rewarding stimulus is encountered and is modulated by physiologic states such as hunger to powerfully motivate behavior. Obese individuals may therefore not, as some have argued, overeat because of an increased sensory pleasure for food but because their levels of wanting food may be altered by years of chronic overconsumption.

6. Rationale for Neuromodulation

The abundance of research into obesity pathophysiology has indicated that it is a complex problem involving multiple brain regions. From an evolutionary perspective, feeding behavior is promoted by redundant neuronal systems, presumably to promote survival in times of food scarcity. As a result, while obesity can result from a number of monogenic alterations [38–42], human obesity is rarely caused by single gene mutations. Instead, increased food availability and palatability promote overeating long after metabolic demands are met. It is therefore unlikely that a single drug or brain region will "cure" all forms of obesity. In addition, animal models of obesity may not have face validity with human feeding behavior due to a lack of incorporation of a cortical control mechanism [21]. Animal models tend to emphasize palatability represented by the fat or carbohydrate content of chow, which on a superficial level may resemble the choices available to a human consumer. However, these models fail to incorporate other aspects of food-seeking behavior including context, variety, and previous exposure to a palatable stimulus (i.e., akin to drug seeking) as powerful influences desire and, ultimately, food consumption. In addition, the discovery of hedonic "hotspots" in animals wherein "liking" and "wanting" in the nucleus accumbens are dissociative properties suggests that increased food intake may occur independently of the pleasurable experience of food consumption [63, 64, 77–79].

The limitations of animal models combined with increased understanding that feeding physiology is complex and relies on input and integration from both homeostatic and hedonic neural circuits argue for further investigation. While neuromodulation in obesity is relatively new, its safety and efficacy profile in thousands of patients with a variety of movement and psychiatric disorders argues for further investigation. While our pilot study [19] did not yield weight loss, it resulted in a decreased urge to eat in all patients, confirming prior animal and human research that the LHA is a central node in the generation of feeding behavior. However, to simply relegate feeding behavior to an "on" or "off" state would be inaccurate as it is now understood that the control of feeding is powerfully regulated by physiologic states and reward valuation [8, 9]. Without adequately addressing the hedonic component of food seeking and the motivational processes that drive eating, any intervention is likely to be unsuccessful.

6.1. Future Perspectives. As demonstrated by our pilot study of LHA-DBS, neuromodulation for obesity is still in its infancy. Future studies of DBS in obesity should enroll a greater number of patients in order to discern the optimal stimulation parameters as well as identify individual patient characteristics that may contribute to the success or failure of DBS as a therapeutic modality for refractory obesity. Additionally, future studies of DBS in obesity should attempt to modulate the reward circuitry either independently or in conjunction with areas such as the LHA and ARC in order to target both essential components of feeding physiology. Arguments for targeting nodes in reward, such as the NAc, stem from the unintended side effects of DBS for other conditions such as OCD in which one patient experienced smoking cessation and another abstinence from alcohol after years of dependence [83, 84]. While obesity and pathological overeating may have some similarities to drug addiction, it should be noted that they have distinct differences [21]. Nonetheless, both addiction and obesity may stem from reward system dysfunction and thus neuromodulation of reward centers may be beneficial in treating obesity.

As alluded to earlier, fMRI imaging has also been performed in obese patients and healthy controls with some suggesting that reward hypofunction may be central to obesity pathophysiology. Imaging studies, such as fMRI, may be used in conjunction with DBS over time to test this hypothesis and to determine whether there are functional differences in patients with obesity that change over time with neuromodulation of brain regions such as the ARC, LHA, and NAc. It is highly likely that DBS studies of the future may also shed light into distinct obesity subtypes that are not recognized today and may have led insight into which patients would benefit most from DBS.

6.2. Ethical Considerations. Opponents of neuromodulation for obesity may argue that it is unethical because it may alter behavior and therefore be compared to psychosurgery. However, in contrast to the ablative nature of the psychosurgical procedures of the past, DBS is slowly becoming an accepted treatment modality for those with intractable psychiatric disease including MDD, OCD, Tourette's, and even addiction. The safety of our pilot study of LHA-DBS and studies for psychiatric disease in which the nucleus accumbens were targeted suggests that neuromodulation is a worthwhile endeavor to better delineate the mechanisms that may be involved in pathological overeating. Investigation into obesity may yield insight into other conditions that have aberrant feeding physiology such as anorexia and bulimia and also may pave the way for expanded applications into disorders such as addiction.

7. Conclusion

In the last several decades, the understanding of feeding physiology has grown considerably more complex. But while the neural networks that govern feeding have become clearer, treatment of pathological overeating that can lead to obesity and obesity-related comorbidities has stalled. In addition, the surgical options (gastric bypass, banding) are available to patients when conservative measures are not without adverse effects. The safety, reliability, of DBS in movement and neuropsychiatric disease encourages investigation in obesity as a potential therapeutic option in patients of whom other forms of treatment have failed.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Influence of Genetic Variants of the N-Methyl-D-Aspartate Receptor on Emotion and Social Behavior in Adolescents

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Considerable evidence has suggested that the epigenetic regulation of N-methyl-D-aspartate (NMDA) glutamate receptors plays a crucial role in neuropsychiatric disorders. Previous exploratory studies have been primarily based on evidence from patients and have rarely sampled the general population. This exploratory study examined the relationship of single-nucleotide polymorphism (SNP) variations in the genes encoding the NMDA receptor (i.e., *GRIN1, GRIN2A, GRIN2B, GRIN2C*, and *GRIN2D*) with emotion and social behavior in adolescents. For this study, 832 tenth-grade Taiwanese volunteers were recruited, and their scores from the Beck Youth Inventories were used to evaluate their emotional and social impairments. Based on these scores, *GRIN1* (rs4880213) was significantly associated with depression and disruptive behavior. In addition, *GRIN2B* (rs7301328) was significantly associated with disruptive behavior. Because emotional and social impairment greatly influence learning ability, the findings of this study provide important information for clinical treatment and the development of promising prevention and treatment strategies, especially in the area of psychological adjustment.

1. Introduction

Interest in the pathology of emotional disorders, such as depression and anxiety, has increased, primarily because the incidence of emotional disorders in adults, adolescents, and even children has dramatically increased over the past several decades [1]. Emotional disorders are often influenced by genetic and lifestyle factors [2, 3], and understanding the genetic etiologies of these diseases could provide valuable information for the development of effective therapies.

The neuronal N-methyl-D-aspartate receptor (NMDAR) has been postulated to play a key role in the pathophysiology of schizophrenia, bipolar disorder, and depression [4, 5]. The possible role of NMDAR signaling in the pathophysiology of emotional disorders has been supported by the following evidence: (a) bipolar disorder and major depression disorder are associated with altered levels of central excitation neurotransmitters [6, 7], (b) NMDAR expression, distribution, and function are decreased in patients with mood disorders [8], (c) the NMDAR modulator exerts a positive therapeutic effect on patients [9], and (d) antidepressants and mood stabilizers can improve NMDAR function [10, 11]. Therefore, genes involved in the NMDAR pathway might be important genetic regulators of human physiology that consequently influence mood diseases.

Recent studies have shown that many of the physiological and pharmacological properties of the NMDAR depend on the composition of its subunits [12]. NMDAR subtypes

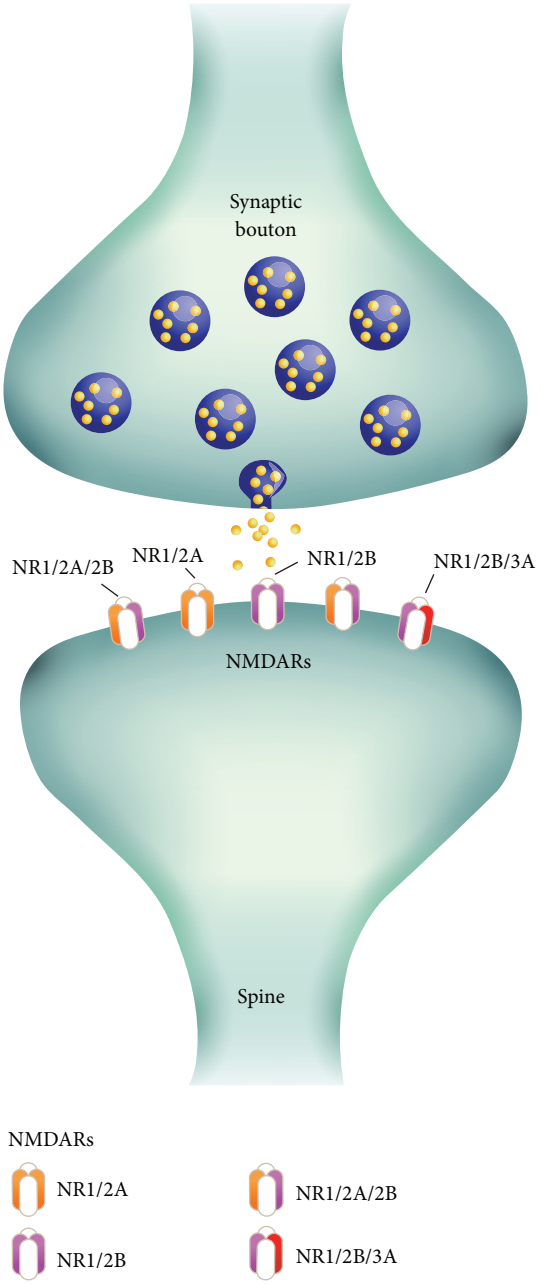

Figure 1: Different NMDA receptor stoichiometric subunits.

include at least one obligatory NR1 subunit in combination with different constellations of NR2 (i.e., GRIN2A, GRIN2B, GRIN2C, and GRIN2D) [13–15] and/or NR3 (i.e., GRIN3A and 3B) subunits. The alternative splicing of the *GRIN1* gene yields eight different NR1 isoforms that form different combinations with NR2 and/or NR3. These isoforms display unique properties in the central nervous system that depend on their subunit composition (Figure 1). The NR1 subunit appears to be a common prerequisite for the expression of functional NMDARs, and the NR2 subunit is required for the efficient formation of functional NMDARs. Moreover, the NR2 subunits determine biophysiological ion channel properties, such as the mean conductance open time and sensitivity to an Mg^{2+} block [16]. The NR2 subunit assembly process may also be a critical factor in postsynaptic signaling pathways that direct synaptic plasticity [17]. The dysfunction of NMDA ligand-gated cation channels is an underling molecular mechanism for neurologic disorders, such as schizophrenia [14], psychiatric disorders [18], and neurodegenerative diseases [19]. Recent studies indicated that alterations in NR1 and NR2 transcript expression associated with the NMDAR stoichiometry in schizophrenia involved more complex cellular changes than previously assumed [20, 21]. The heteromeric nature of NMDARs provides a wide variety of receptor subtypes; thus, single-nucleotide polymorphisms (SNPs) in NMDAR subunits are likely responsible for creating various distinct NMDAR properties. Several SNPs in the NMDAR subunit genes have been shown to affect the role of NMDAR signaling in the pathophysiology of mood disorders by altering the expression, distribution, and function of the NMDARs [4, 22].

Two SNPs of *GRIN1* merit special attention: rs11146020 (G1001C) and rs4880213 (*GRIN1* 5′-upstream region). The *GRIN1* (rs11146020) gene is located at 9q34, a locus in the promoter region of the NMDAR subunit that has been linked to schizophrenia [23, 24]. The *GRIN1* (rs11146020) gene product plays a fundamental role in many brain functions, and its involvement in the pathogenesis of schizophrenia has been widely investigated [25–27]. Only a few studies have focused on *GRIN1* rs4880213 and the pathogenesis of schizophrenia or mood disorders. Two studies showed that individuals with the *GRIN1* rs4880213 C/C variation displayed more severe disability and reduced NMDAR-mediated cortical excitability than individuals with the C/T or T/T variation [28, 29].

Further studies revealed that variations in the NMDAR 2B subunit gene (*GRIN2B*) were associated with schizophrenia, psychiatric disorders, and brain plasticity. For example, in human attention performance studies, subjects that were homozygous for the frequent C allele of the *GRIN2B* rs1806201 variation displayed more altered network scores than subjects in the other two genotype groups (C/T and T/T) [30]. The other 2B subunits of NMDAR have promising candidate SNP variants (i.e., rs1805476, rs1805477, rs1805501, and rs1805502) that affect the genetic susceptibility to obsessive-compulsive disorder [31]. In a functional study of protein expression, the *GRIN2B* rs1805502 (T5988C) C allele was associated with reduced *GRIN1* mRNA and protein expression in schizophrenic patients [5]. In pharmacological interventions, the same variant of NMDAR (*GRIN2B*) has been proposed to act as a genetic predictor of treatment-resistant depression (TRD) in patients with major depressive disorder (MDD) [32].

Understanding the involvement of *GRIN* genes in neurocognitive deficits will shed light on the importance of links between subunit involvement in plasticity paradigms and behavior. However, most previous studies of SNP associations with schizophrenia only investigated one or two genes in NMDARs, particularly *GRIN1*, *GRIN2A*, and *GRIN2B* [5, 24, 33]. Therefore, in this study, we used more extensive systemic sequencing to identify SNPs that may affect multiple genes

related to NMDARs. Our results further elucidate the role of NMDAR signaling in emotion and social behavior.

This exploratory study examined the relationship of SNP variations in NMDAR genes with emotion and social behavior in adolescents. Data from randomized controlled trials could provide convincing evidence on the mechanisms of genetic and epigenetic effects on emotion [34]. However, previous exploratory studies have been primarily based on evidence from patients and have rarely sampled the general population. In addition, previous studies of schizophrenia revealed that the total number of NMDARs remains normal as the patient ages, but the receptor stoichiometry appears to change with age [35]. Therefore, an investigation of the impact of epigenetic changes on emotion and behavior in different cross sections of the population could be important for understanding the underlying mechanisms of mood disorders. To the best of our knowledge, the association between genetic SNPs and emotion in adolescents has not yet been investigated. This study aimed to fill this gap. Furthermore, epidemiological studies have linked mood disorders to gender differences. Specifically, women are more likely than men to be diagnosed with depression [36, 37]. Therefore, the current study aimed to control for the influence of gender on the effect of genetic polymorphisms on emotion using statistics.

2. Materials and Methods

2.1. Participants. Three public senior high schools were selected (one in southern Taiwan, one in central Taiwan, and one in northern Taiwan). A total of 832 tenth-grade volunteers, 269 of whom were male, were recruited for this study. The mean age of the subjects was 16.3 years (SD: 0.5; age range: 16-17 years). The volunteers and their parents were explicitly informed about the plan, protocol, and procedure for the study, and written consent was obtained prior to the start of the study. This study was approved by the institutional review board of the National Taiwan University Hospital and the ClinicalTrials.gov registry of clinical trials (ClinicalTrials.gov identifier: NCT00713570).

2.2. Gene Screening, Variation Analysis, and Bioinformatics. We investigated the association of 59 SNPs in the NMDAR genes (i.e., *GRIN1*, *GRIN2A*, *GRIN2B*, *GRIN2C*, and *GRIN2D*) with emotion and behavior in 832 Han Chinese subjects. Fifty-nine SNPs from the entire set of candidate genes associated with NMDARs were identified based on information available in the Entrez Gene (http://www.ncbi.nlm.nih.gov/gene), HapMap (http://www.hapmap.org), and Ensembl (http://www.ensembl.org/Homo_sapiens) databases. In a pilot study, we analyzed genotypes by sequencing DNA from 20 subjects, and we assumed that some genetic polymorphisms involved in functional NMDAR subunits might contribute to individual differences in emotion and social behavior (GSJUNIOR_A16, Roche Union ClinBio Co. Ltd.). SNPs with a minor allele frequency greater than 5% in the pilot study that were identified in the analyzed samples were considered the most promising candidates, and representative common variants were selected. From the pilot study, 8 SNPs from the *GRIN1* and *GRIN2* genes were identified as the most promising candidates. The samples were genotyped by DNA sequencing of the relevant PCR products using an ABI Prism_ BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit and an ABI Prism_3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All participants were screened for the 8 selected SNPs using a custom/commercial TaqMan SNP genotyping assay according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA) and a ViiA 7 real-time PCR system (Life Technologies).

2.3. Emotion Assessment. The subjects' emotional behaviors were assessed via the second edition of the complete Beck Youth Inventories (BYI-II) [38], which is a commonly used and thoroughly standardized test that suitably assesses the diversity of emotion and behavior in adolescents. The BYI-II consists of five inventories: anxiety, depression, anger, disruptive behavior, and self-concept, and each of these inventories contains 20 items that are assessed based on five self-reported scales. Higher scores for the inventories of anxiety, depression, anger, or disruptive behavior indicate a tendency towards emotional instability. In contrast, a higher score in the self-concept inventory reveals that the student has a positive sense of self.

2.4. Statistical Analysis. Two-tailed t-tests were used to assess the significance of differences in the BYI-II subtask scores between males and females. For each SNP, the participants were assigned to one of three groups based on their genotype, and deviation from the Hardy-Weinberg equilibrium was tested using a chi-squared test. An analysis of covariance (ANCOVA) was employed to determine the effect of each SNP genotype on behavior based on the BYI-II subtask scores. Previous studies revealed that gender is an important determinant of emotion. To assess the effect of genetic polymorphism on emotion, the gender of participants served as a covariate in this analysis to control for gender effects on BYI-II subtask scores. Post hoc analyses using pairwise comparisons were also used to identify trends in the data. The level of confidence was set at the 0.05 significance level. In consideration of type I errors, permutation tests (with N = 1,000 randomizations) [39] were performed to correct for multiple comparisons. The assumptions used for the ANCOVA and inferential statistical analyses were tested using SPSS version 22.0.

3. Results

NMDARs are tetramers that contain two GRIN1 subunits bound to two GRIN2 (A, B, C, or D) and/or GRIN3 (A or B) subunits. Many of the physiological and pharmacological properties of NMDARs depend on the specific GRIN2 and GRIN3 subunits. The observed distributions of the genotypes for each SNP appeared to follow Hardy-Weinberg equilibrium. The analyses of SNPs in NMDAR-related genes, genotypic distribution, chromosome localization, and genotype frequency are presented in Table 1.

Table 1: The genotype distributions and chromosome locations of the SNPs.

Gene/SNP ID	Allele/genotype	Subjects	Chromosome region	Genotype frequency	CHB population frequency*
GRIN1			9q34.3		
rs4880213	CC/CT/TT	124/368/340		0.15/0.44/0.41	0.11/0.44/0.45
rs11146020	CC/CG/GG	565/239/28		0.68/0.29/0.03	0.72/0.24/0.04
GRIN2B			12p12		
rs7301328	CC/CG/GG	250/396/186		0.30/0.48/0.22	0.26/0.43/0.31
rs1806201	AA/AG/GG	223/438/171		0.27/0.53/0.20	0.22/0.55/0.22
rs1805247	AA/AG/GG	608/204/20		0.73/0.25/0.02	0.61/0.35/0.04
rs1805502	AA/AG/GG	608/203/20		0.73/0.24/0.03	0.61/0.35/0.04
rs3764028	AA/AC/CC	148/397/287		0.18/0.48/0.34	0.13/0.46/0.42
GRIN2C			17q25		
rs3744215	AA/AC/CC	143/423/266		0.17/0.51/0.32	0.15/0.47/0.38

*Data from 1000 Genomes Project Phase 3; http://www.1000genomes.org/.

Table 2: Distribution of selected characteristics of the participants.

	Male	Female	*P*
Age (yrs)	16.8 (0.32)*	16.8 (0.30)	
BYI-II			
Anxiety	16.2 (8.9)	18.5 (8.8)	<0.01
Depression	13.6 (9.4)	15.1 (9.3)	
Anger	11.7 (8.9)	12.4 (7.8)	
Disruptive behavior	8.2 (7.2)	6.6 (4.9)	<0.01
Self-concept	36.0 (9.6)	36.5 (7.9)	

*Mean (SD).

The demographic and BYI-II subtask scores of the participants are outlined in Table 2. Some differences between males and females were observed. Female subjects in this study tended to be more anxious, whereas male subjects exhibited more disruptive behaviors.

As shown in Table 3, the emotional instability significantly differed in individuals carrying the *GRIN1* (rs4880213) and *GRIN2B* (rs7301328) polymorphisms. After the multiple comparison correction by 1,000 permutation tests, *GRIN1* (rs4880213) was significantly associated with depression ($P = 0.04$) and disruptive behavior ($P = 0.04$), and *GRIN2B* (rs7301328) was significantly associated with disruptive behavior ($P = 0.05$). The other six SNPs were not significantly associated with behavior based on the BYI-II analysis.

The ANCOVA of the depression subtask scores revealed a significant main effect for the three genotype groups of *GRIN1* (rs4880213; Table 4, $F[2, 829] = 4.5$, $P < 0.05$). The pairwise test revealed that the depression subtask scores of the T/T genotype group were significantly lower than those of the C/C and C/T genotype groups (Table 5, C/C > C/T, $P = 0.005$; C/T > T/T, $P = 0.04$). The *GRIN1* (rs4880213) polymorphisms also influenced the disruptive behavior scores (Table 6, $F[2, 829] = 4.0$, $P < 0.05$). Subjects with the T/T genotype group tended to score lower on the disruptive behavior subtask (Table 7, C/T > T/T, $P = 0.005$). Furthermore, the disruptive behavior scores were significantly associated with the *GRIN2B* rs7301328 polymorphism (Table 8, $F[2, 829] = 4.2$, $P = 0.05$). As shown in Table 9, a pairwise comparison analysis revealed that the disruptive behavior subtask scores of the G/G genotype group were significantly lower than those of the C/C genotype group ($P = 0.004$).

4. Discussion

All NMDARs appear to function as heteromeric assemblies that consist of multiple NR1 subunits and at least one type of NR2. The NR3 subunit does not form functional receptors alone but can coassemble with NR1/NR2 complexes. The temporal and spatial expression patterns of the NR2 and NR3 subunits were recently shown to differ in the brain, and the expression of NMDAR subtypes also vary by cell type and subcellular localization [40, 41]. *In situ* hybridization studies have shown that the mRNAs for NMDAR subunits are differentially distributed throughout the brain, and the expression patterns of these mRNAs change strikingly during development [42]. This study aimed to determine whether any other SNPs are responsible for the distinct properties of the NMDAR that are associated with emotion and behavior, especially in adolescents. Previous studies show that *GRIN1* (rs11146020) was strongly associated with bipolar disorder [4] and depressive symptoms [43]. The incidences of depression and disruptive behavior were significantly lower in participants that carried the *GRIN1* T/T genotype (SNP rs4880213) than in members of the two other groups; this result was consistent with Georgi's study, which showed that the T allele was less frequent in schizophrenia patients with a lifetime history of depression than in control. Francesco et al. [44] suggested that the T/T genotype of the *GRIN1* rs4880213 SNP is associated with reduced intracortical inhibition, enhanced glutamatergic excitation, and enhanced glutamate NMDAR function. Francesco's study also revealed that the *GRIN1* and *GRIN2B* subunits of NMDARs are involved in regulating cortical excitability and plasticity in the human cortex. Rossi et al. [28] also indicated that the C allele of rs4880213 is associated with reduced NMDAR-mediated cortical excitability. Notably, a number of studies have indicated that NMDAR dysfunction is associated with depression syndrome [10]. Thus, the homozygosity of the *GRIN1* rs4880213 T allele might

Table 3: Genotype distributions and comparisons of subtask scores and genotype groupings.

	C/C	C/T	T/T	F	P_{cor}
GRIN1 rs4880213					
Anxiety	19.1 ± 9.5*	18.2 ± 9.0	17.0 ± 8.5	2.6	>0.05
Depression	16.5 ± 9.8	15.0 ± 9.5	13.5 ± 8.8	4.6	**0.04**
Anger	12.3 ± 7.8	12.8 ± 8.3	11.6 ± 8.0	1.4	>0.05
Disruptive behavior	7.2 ± 5.6	7.7 ± 6.1	6.4 ± 5.1	4.1	**0.04**
Self-concept	36.0 ± 7.9	36.2 ± 9.2	36.7 ± 8.0	0.4	>0.05
	C/C	C/G	G/G		
GRIN2B rs7301328					
Anxiety	17.5 ± 8.6	17.8 ± 9.0	18.6 ± 9.3	0.8	>0.05
Depression	14.1 ± 8.9	14.6 ± 9.1	15.5 ± 10.5	1.1	>0.05
Anger	11.6 ± 7.8	12.3 ± 7.8	13.1 ± 9.2	1.5	>0.05
Disruptive behavior	6.3 ± 5.6	7.2 ± 5.6	7.9 ± 5.8	3.7	**0.05**
Self-concept	36.2 ± 8.9	36.9 ± 7.6	35.7 ± 9.5	1.2	>0.05

*Mean values ± standard deviations.
P_{cor}: multiple comparison correction by 1,000 permutation tests.

Table 4: ANCOVA summary for the depression subtask scores.

Source of variance	Df	SS	MS	F	P
GRIN1 rs4880213	2	787.6	393.8	4.5	0.04*
Gender	1	278.3	278.3	3.2	0.07
Residual	828	21794	26.3		

*Multiple comparison correction by 1000 permutation tests.

Table 5: Pairwise comparisons of three *GRIN1* rs4880213 genotype groups in terms of adjustments of depression subtask scores.

GRIN1 rs4880213	M_{adj}	SE	Pairwise comparison
C/C	16.5	0.9	C/C > T/T (P = 0.005)
C/T	15.1	0.5	C/T > T/T (P = 0.04)
T/T	13.5	0.5	

Table 6: ANCOVA summary for the disruptive behavior subtask scores.

Source of variance	Df	SS	MS	F	P
GRIN1 rs4880213	2	254.3	127.1	4.0	0.04*
Gender	1	335.2	335.2	10.5	0.001
Residual	828	21794	26.5		

*Multiple comparison correction by 1,000 permutation tests.

Table 7: Pairwise comparisons of three *GRIN1* rs4880213 genotype groups in terms of adjustments of disruptive behavior subtask scores.

GRIN1 rs4880213	M_{adj}	SE	Pairwise comparison
C/C	7.3	0.5	C/T > T/T (P = 0.005)
C/T	7.7	0.3	
T/T	6.4	0.3	

Table 8: ANCOVA summary for the disruptive behavior subtask scores.

Source of variance	Df	SS	MS	F	P
GRIN2B rs7301328	2	269.2	269.2	4.2	0.05*
Gender	1	386.3	386.3	12.1	0.001
Residual	828	21779	26.3		

*Multiple comparison correction by 1,000 permutation tests.

Table 9: Pairwise comparisons of three *GRIN2B rs7301328* genotype groups in terms of adjustments of disruptive behavior subtask scores.

GRIN2B rs7301328	M_{adj}	SE	Pairwise comparison
C/C	6.2	0.5	C/C > G/G (P = 0.004)
C/G	7.2	0.3	
G/G	8.0	0.3	

increase glutamate NMDAR function and stabilize the mood status compared with other alleles. However, because the effect of the *GRIN1* rs4880213 SNP on NMDAR function has not yet been analyzed at the molecular or metalevel, the mechanism by which the *GRIN1* rs4880213 SNP affects NMDAR function needs to be investigated.

Our results also showed that the homozygosity of the C allele of the *GRIN2B* (rs7301328) is associated with increases in disruptive behavior; this finding is consistent with a report by Ohtsuki et al. [45], which showed that the G allele of the 366C/G (rs7301328) polymorphism is more common in patients than in population-based controls. The *GRIN2B* SNP (rs7301328) has been linked to bipolar disorder [24], schizophrenia, and other neuropsychiatric disorders; however, to the best of our knowledge, few studies have investigated the influence of the *GRIN2B* SNP (rs7301328) at the molecular level, including its effect on NMDAR function or on the level of *GRIN1* or *GRIN2* expression. In addition, the currently available studies do not conclusively show that unique *GRIN2* subunits selectively mediate directions of plasticity, especially regarding long-term depression (LTD). In some studies, behavioral impairment can be related to

a selective deficit in one direction of plasticity. Moreover, a *GRIN2B*-dependent LTD-like process has also often been implicated in mechanisms that support reversal learning [4, 46]. A rigorous testing of this hypothesis based on an analysis of more complete information concerning the effect of the *GRIN2B* SNP (rs7301328) would be interesting.

This study did not identify significant associations between the other polymorphisms and behavioral indices. Previous exploratory studies have indicated that *GRIN1* (rs11146020) is a good candidate for susceptibility to schizophrenia [24, 25, 33], and other studies have suggested that the combined effects of *GRIN1* and *GRIN2B* gene polymorphisms, including rs1805247 and rs1805502, might be involved in the etiology of schizophrenia. However, we did not identify such an association. Notably, the students in this study were physically and psychologically healthy, and identifying significant differences in emotional performance among different genotype groups is more difficult in a healthy population than in patients who are suffering from single-gene diseases or psychiatric disorders.

Here, we report an exploratory study of SNPs in the NMDAR *GRIN1* and *GRIN2* subunit genes in a healthy adolescent population. Specifically, we verified that the two SNPs (rs4880213 and rs7301328) influenced emotional performance in this adolescent population. Nevertheless, this study is subject to some limitations. First, most of the participants were relatively physically healthy. Nevertheless, the effect size for the association between the examined SNPs and behavior was narrow. However, because observing significant differences within a normal population is difficult, future studies may rely on the data presented herein to explore mechanisms relevant to this association, both at the clinical and molecular levels. The other limitation of the present study is differences in the socioeconomic levels of students which may have influenced the emotional behavior assessments. All volunteers in this study lived in metropolitan areas of Taiwan. Studies showed students socioeconomic levels were likely approximately equivalent in metropolitan areas of Taiwan [47]. Therefore, the impact of social economy on emotion in this study might be less significant. However, because the current study did not measure the effects of socioeconomic status on emotional behavior, we cannot conclusively rule out such an effect. Subsequent studies should rigorously examine this effect.

Educational researchers, teachers, and counselors might be interested in the implications of the effects of genetic polymorphisms identified herein. Specifically, students who do not perform as well as others academically due to poor cognitive abilities or emotional self-control show a decreased willingness to learn. Genotyping these students could provide educators with a strategic understanding of the potential innate emotional self-control of an individual student. Because human emotion is influenced by interactions between genetic variations and environmental factors, this knowledge could be used to provide an appropriate environment and monitor the emotions of a student during learning. These strategies could improve a student's interest in learning and achievement.

Conflict of Interests

None of the authors have any conflict of interests to report.

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Research Article

A High-Fat Diet Causes Impairment in Hippocampal Memory and Sex-Dependent Alterations in Peripheral Metabolism

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While high-fat diets are associated with rising incidence of obesity/type-2 diabetes and can induce metabolic and cognitive deficits, sex-dependent comparisons are rarely systematically made. Effects of exclusive consumption of a high-fat diet (HFD) on systemic metabolism and on behavioral measures of hippocampal-dependent memory were compared in young male and female LE rats. Littermates were fed from weaning either a HFD or a control diet (CD) for 12 wk prior to testing. Sex-different effects of the HFD were observed in classic metabolic signs associated with type-2 diabetes. Males fed the HFD became obese, and had elevated fasted blood glucose levels, elevated corticosterone, and impaired glucose-tolerance, while females on the HFD exhibited only elevated corticosterone. Regardless of peripheral metabolism alteration, rats of both sexes fed the HFD were equally impaired in a spatial object recognition memory task associated with impaired hippocampal function. While the metabolic changes reported here have been characterized previously in males, the set of diet-induced effects observed here in females are novel. Impaired memory can have significant cognitive consequences, over the short-term and over the lifespan. A significant need exists for comparative research into sex-dependent differences underlying obesity and metabolic syndromes relating systemic, cognitive, and neural plasticity mechanisms.

1. Introduction

The global prevalence of high-fat diets has led to an epidemic of obesity, insulin-resistance, and type 2 diabetes. Only one-third of American adults are of "normal" weight. One-third of children presenting with diabetes are diagnosed as type 2 [1], a clinical diagnosis formerly rare in children, and often attributed to obesity-induced insulin-resistance. Systematic studies assessing a comprehensive range of systemic metabolic, cognitive, and neuronal deficits linked to diet-induced glucose dysregulation are rare, while studies assessing these domains in reproductively normal females are nearly nonexistent. In the US, the NIH has recently mandated the inclusion of both males and females in clinical research, directing attention to the importance of sexual dimorphism in disorders where sex as a variable has been largely ignored.

Dietary induced obesity has previously been shown to impair performance in a spontaneous alternation task, a measure of hippocampal-dependent spatial memory, while administration of intrahippocampal insulin improved performance [2]. It has been well documented that rats with hippocampal lesions are impaired in a variety of spatial learning tasks requiring integration and use of environmental cues [3–9]. One such task is spatial object recognition, where successful memory is assayed by the relative amount of time that a subject spends with a familiar object moved to a novel location during testing, that is, recognition of the object's change in spatial location between trials. Rodents with hippocampal damage are unable to successfully recognize the moved object [10].

Animals fed high-fat diets show significant potentially pathological changes in hippocampus, including reduced dendritic spines in CA1 [11] and impaired LTP [12] along with memory impairment. Comprehensive comparisons of diet-induced systemic dysregulation of glucose control and of

concordant impairment of cognitive function, that is, in both males and females, are needed but unfortunately rare.

The experiments presented here address major sex-dependent diet-induced alterations in systemic metabolism, along with sex-independent severe cognitive (memory) deficits, of rats fed a high-fat diet (HFD) compared to littermates fed a control diet (CD) from weaning.

2. Materials and Methods

2.1. Subjects. Young adult littermate Long-Evans (LE) outbred rats were socially housed on a 12 hr light/dark cycle with *ad libitum* access to food and water according to their assigned diet, with different diet cohorts housed in different cages from weaning (3 wk of age). Daily records of weight were maintained throughout the study. All procedures were conducted with approval of the Institutional Animal Care and Use Committee of the University of Texas at Dallas in accordance with the guidelines of the USDA.

2.2. Diet. All subjects were fed from weaning their assigned diet for 12–15 wk prior to physiological and behavioral assessment. Control diet (CD) groups received 14% fat, 64.8% carbohydrate, and 21.2% protein rat chow (Open Source Diets) along with pure filtered water. High-fat diet (HFD) groups received 58% fat, 25.5% carbohydrate, and 16.4% protein rat chow (Open Source Diets chow augmented with saturated fat (coconut oil) and casein protein) to induce metabolic changes. Nutritional sufficiency of the modified data was assayed and confirmed by Open Source Diets.

2.3. Fasting Blood Glucose. Prior to testing, subjects were fasted overnight (8–10 h) to deplete glycogen stores and reduce baseline variability between subjects. Blood samples were obtained by tail nick from well-handled behaviorally naive rats in cohorts of 4 to 8 males and females. Subjects were handled for 1 h prior to testing to reduce stress related fluctuations in blood glucose. Blood glucose levels (mg/dL) were assessed with an AlphaTRAK whole-blood glucose monitor (Abbott Laboratories) and AlphaTRAK 2 test strips. Calibration of the glucose meter was confirmed weekly using AlphaTRAK 2 control solution.

2.4. Oral Glucose-Tolerance Testing (GTT). Glucose-tolerance testing was performed to assess a primary symptom of type 2 diabetes. Again, prior to testing, subjects were fasted overnight (8–10 h) to deplete glycogen stores and reduce baseline variability between subjects. Subjects were handled for 1 h prior to testing to reduce stress-related fluctuations in blood glucose. Basal blood glucose levels (mg/dL) were obtained via tail nick with an AlphaTRAK whole-blood glucose monitor (Abbott Laboratories). Subjects then immediately received either an oral bolus of glucose (2 g/kg) or 0.9% of saline via intragastric lavage tube, and blood glucose was assessed every 15 min for 120 min (in well-handled rats, delivery of saline produced no significantly different fluctuation in serum glucose comparing between oral and i.p. methods of infusion ($p = 0.9$), so responses to saline in the GTT and ITT experiments were combined within (but not between) each of the four groups tested).

2.5. Insulin-Tolerance Testing (ITT). Insulin-tolerance testing was performed using the same protocol described for GTT; however, subjects received either an intraperitoneal (i.p.) bolus injection of insulin (1 U/kg) or 0.9% of saline after baseline readings were obtained (in well-handled rats, delivery of saline produced no significantly different fluctuation in serum glucose comparing between oral and i.p. methods of infusion ($p = 0.9$), so responses to saline in the GTT and ITT experiments were combined within (but not between) each of the four groups tested).

2.6. Plasma Corticosterone, Leptin, and Estradiol Analyses. Plasma samples were aliquoted into 500 μL tubes and frozen until use to avoid repeated freeze/thaw cycles. The plasma was thawed at room temperature for 1 hr and then diluted appropriately for ELISA assays. Corticosterone (CORT) was assessed via corticosterone (CSCI) ELISA kits (Abcam), leptin was assessed via rat leptin ELISA kits (Crystal Chem), and estradiol was assessed via mouse/rat estradiol ELISA kits (Calbiotech), using an ELx800 plate reader (BioTek) with Gen5 software.

2.7. Spatial Object Recognition (SOR) Testing. All behavioral experimentation was carried out in a white plywood box 60 × 60 × 60 cm with 15 × 15 cm gridlines on the bottom (see Figure 6(a)). Objects were identical solid, nonporous, 5 cm aluminum cubes. Large visual cues were located on adjacent walls of the apparatus for spatial orientation. To minimize odor cues, the apparatus and objects were cleaned first with 30% ethanol and then with Micro90 enzymatic cleaner (International Products Corp.) both before testing began and between all trials. The apparatus was in a room without windows, and dim diffuse overhead lighting was used to avoid shadows.

The following protocol was used to assess SOR (see Figures 6(b) and 6(c)). Rats were handled for 7–10 d prior to 3 d of habituation to the apparatus. On testing day, rats were placed into the apparatus with 2 objects in 2 different locations and allowed to explore for 5 min. After a 30 min intertrial interval (ITI), rats were returned to the apparatus for testing and allowed to explore a duplicate set of objects, with 1 moved to a novel location and 1 placed in a familiar location (identical locale to one of the original objects). Behavior was observed remotely via video recordings, and time spent exploring each object was measured. Behavioral videos were independently scored by two or more assistants blind to the experimental groups to avoid bias, with interrater reliability scores consistently >0.97.

2.8. Analyses. One-way ANOVAs with repeated measures (values corrected with Tukey's test) were performed using Prism 6 (GraphPad). Data are presented graphically as means ± SEM, with individual data scatter included in some graphs for additional clarity. Numbers of subjects tested for each measure are also shown in each graph.

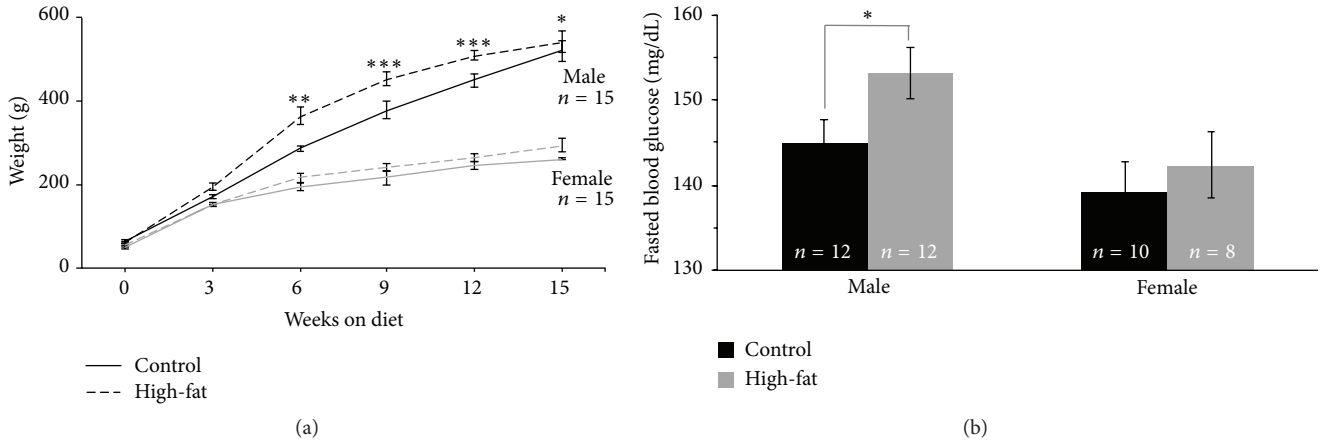


Figure 1: Sex-dependent effects of high-fat diet on body mass and on fasted blood glucose. (a) Males on the HFD gained significantly more weight by week 6 and continued to do so for the remainder of the experiment compared to their CD counterparts. After 15 wk on the diets, control fed males outweighed control fed females by 44%, while HFD fed males outweighed HFD fed females by 50%. Ingesting the HFD did not significantly increase female body weight ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). (b) Male HFD fed rats had significantly elevated fasting blood glucose compared to both control males and HFD females ($p = 0.05$), while no differences were observed between the dietary groups in female rats.

3. Results and Discussion

A total of 21 young-adult male rats fed the CD and 21 young-adult male rats fed the HFD from weaning along with 20 young-adult female rats fed the CD and 19 young-adult female rats fed the HFD from weaning generated the data presented here. Again, all diet- and sex-dependent effects were assessed in matched littermate cohorts (i.e., both males and females from the same litters were assigned to each treatment condition to reduce variance), with results reported as means ± SEM.

3.1. HFD Fed Males, but Not Females, Became Obese. All rats tested gained weight steadily on both diets across the span tested ($F(15, 108) = 23.31$, $p = 0.0001$), with males exhibiting more dramatic weight gains on both diets (see Figure 1(a)). Notably, male rats on the HFD gained significantly more weight than their littermates fed the CD: HFD males had significantly greater body mass than controls on week 6 ($p = 0.004$), week 9 ($p = 0.0001$), week 12 ($p = 0.0001$), and week 15 ($p = 0.05$). However, female rats on the HFD did not gain significantly more weight than their littermates fed the CD. By week 15, males fed the HFD outweighed CD males by 13%, while HFD females outweighed CD females by only 4%. Previous studies suggest that obesity is influenced by sex hormones; female rats gain less weight compared to males when fed a high-fat diet, but this difference is no longer seen after ovariectomy [13]. Our own data (see Figure 4(b)) do not indicate significant alterations in circulating estradiol or in cycle-dependence of subsequent behavioral performance (data not shown) in females fed a HFD. Within a relatively short time on the HFD (3 mo), males became obese while females did not. Since LE rats are an outbred strain, these data may be of greater comparative value than that obtained in studies using inbred strains of rats or mice.

3.2. HFD Fed Males, but Not Females, Had Elevated Fasting Blood Glucose. According to the American Diabetes Association [14], one major criterion for diagnosis of type 2 diabetes in human patients is a significant elevation of fasting blood glucose. Basal fasting blood glucose concentrations (i.e., prior to additional external challenges) sampled from HFD versus CD rats were significantly different in males (see Figure 1(b), $F(3, 36) = 3.42$, $p = 0.05$), with significant elevations in the HFD group compared to controls. However, fasting basal blood glucose was not significantly different in female rats comparing between the two diet groups ($p = 0.2$). Total endogenous circulating glucose has been shown to be greater in men than women due to differences in general body size between sexes [15, 16]. Therefore the body mass (weight) differences discussed previously may contribute to these differences seen in fasted blood glucose levels. As described below (Figures 2 and 3, data from males and females, resp.), when control of circulating blood glucose was challenged, either with a bolus injection of glucose or with a bolus injection of insulin, further sex- and diet-dependent differences were observed.

3.3. HFD Impairs Male, but Not Female, Systemic Glucose-Tolerance. Rats on the HFD exhibited sex-dependent alterations in systemic blood glucose responses to a bolus oral infusion of glucose. Compared to physiological saline infusion, blood glucose was significantly elevated in all groups (i.e., males and females, irrespective of diet) tested 15 min after glucose infusion ($F(16, 112) = 3.758$, $p = 0.001$). Sex- and diet-dependent differences in the later sustained magnitude and duration of responses to this glucose challenge (glucose-tolerance) are detailed below.

Blood glucose of male CD rats after an oral bolus of glucose rapidly returned to baseline after its initial rise (see Figure 2(a)) and was not significantly elevated compared to

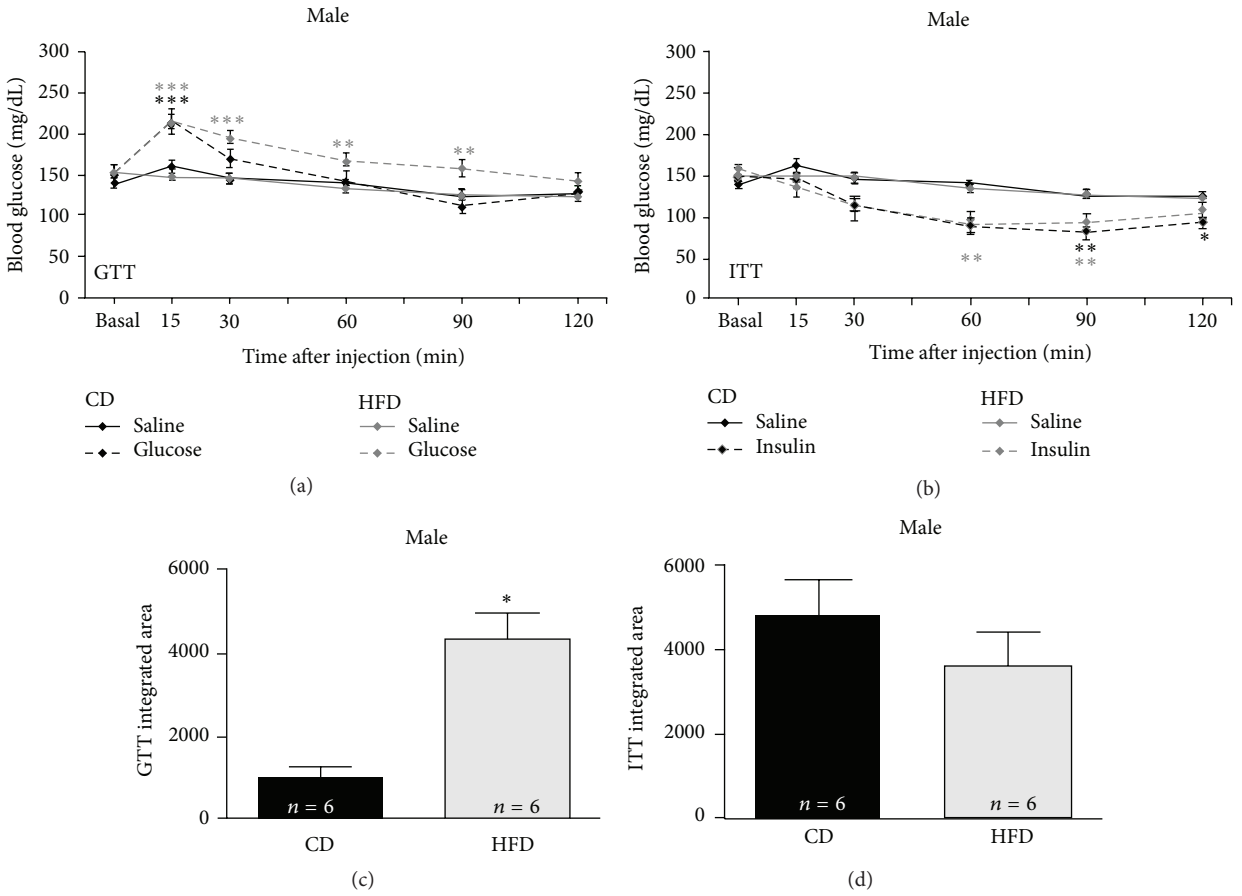


Figure 2: HFD diet leads to failure of GTT in male rats. Glucose-tolerance testing (a) is used clinically as a diagnostic tool for diabetes, while insulin-tolerance testing (b) is informative but not widely used clinically. Comparisons between fasted male rats previously fed the CD or the HFD for 15 wk showed significant differences in blood glucose regulation after an oral bolus of glucose between these diet groups (a). Controls showed a normal rapid rise and fall of blood glucose (significantly elevated only at 15 min after ingesting the glucose bolus), while HFD fed male rats showed significant elevations of blood glucose for 2 hr after glucose bolus. Insulin-tolerance testing showed small diet-dependent differences in male rats, with a faster return to baseline after a bolus injection of insulin in HFD fed males (b), suggestive that at this age HFD males not only remained systemically sensitive to insulin but can successfully utilize exogenous insulin in glucose regulation. Integrated area under the GTT curve (c), but not over the ITT curve (d), was also significantly increased in HFD fed males ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

oral saline infused male CD rats at intervals 30 min or more after infusion ($p = 0.2$). Blood glucose of male HFD rats after an oral bolus of glucose failed to rapidly return to baseline (see Figure 2(a)) and was significantly elevated compared to saline infused male HFD rats at intervals 30 ($p = 0.001$), 60 ($p = 0.01$), and 90 ($p = 0.01$) min after injection. Such a sustained elevation of blood glucose would constitute a failed glucose-tolerance test in clinical assays for type 2 diabetes.

Blood glucose of female CD rats after an oral bolus of glucose more slowly returned to baseline after its initial rise (see Figure 3(a)) and was significantly elevated compared to saline infused female CD rats 30 min after the bolus ($p = 0.05$), returning to baseline 60 min or more after infusion ($p = 0.1$). Unlike in males, blood glucose of female HFD rats after an oral bolus of glucose was only slightly less elevated than that of CD female rats infused with glucose 15 min after injection ($p = 0.08$). Blood glucose of female HFD rats after an oral bolus of glucose returned to baseline more rapidly (see Figure 3(a)) than CD females and was not significantly elevated, compared to saline injected female HFD rats, at intervals 30 min or more after infusion. Although responses of females to glucose challenge (glucose-tolerance) were not identical to those of male, HFD females maintained homeostatic control of circulating glucose in a manner similar to that of female controls.

An additional form of analysis, integrated area under the curve (AUC, a measure of total blood glucose elevation across the entire glucose-tolerance test interval, referenced to the equivalent group's saline-infusion curves) was significantly different between groups tested [$F(3, 16) = 6.048$, $p = 0.006$]. No sex-dependent differences in AUC were observed for controls: AUC was not significantly greater for CD females compared to CD males ($p = 0.3$). However, sex-dependent differences in AUC were observed for HFD rats. AUC was

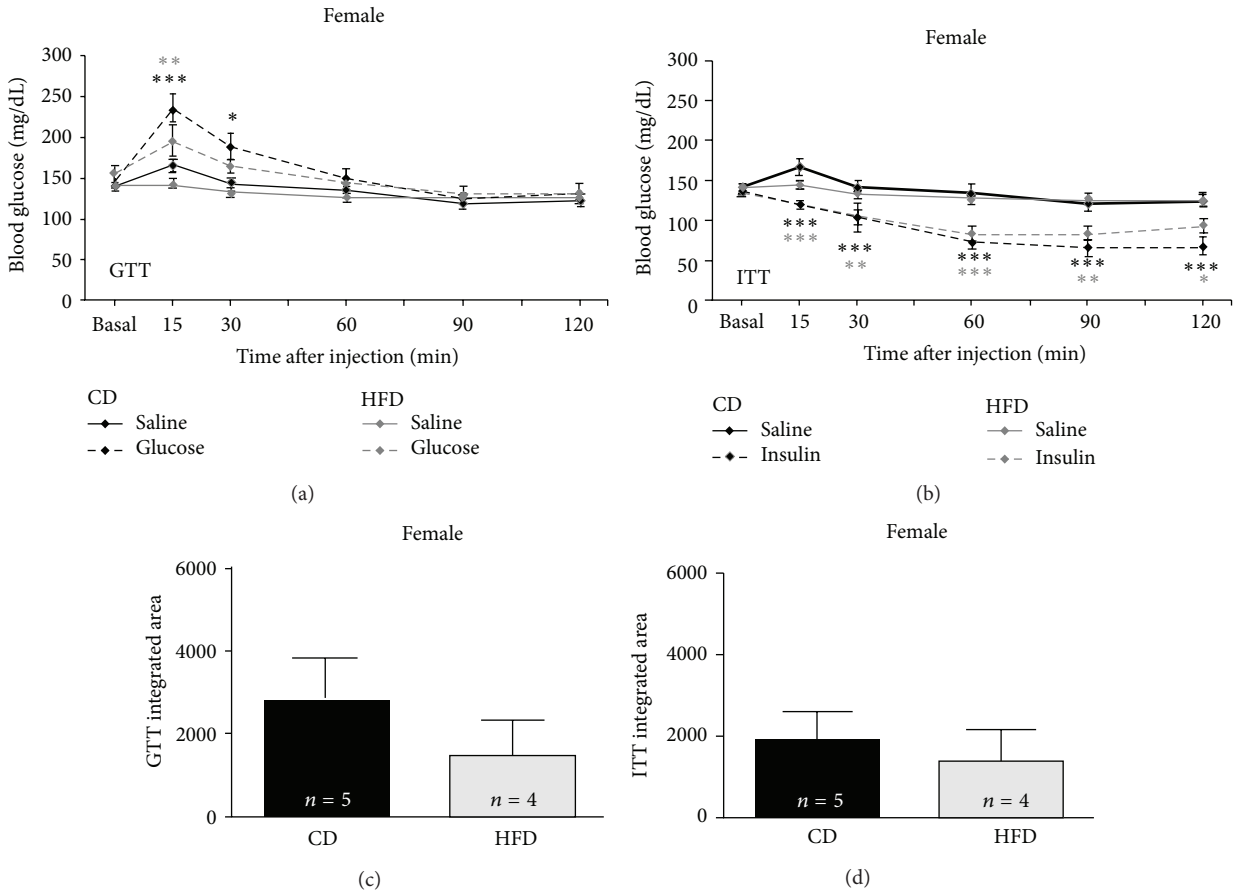


Figure 3: HFD did not significantly alter GTT or ITT in female rats. In contrast to males, female rats on CD and HFD exhibited relatively little diet-dependent changes in glucose- (a) or insulin-tolerance (b). Additionally, no dietary effects were seen in the integrated area under or above the curve (resp.) for either glucose-tolerance testing (c) or insulin-tolerance testing (d).

significantly greater in HFD males compared to HFD females ($p = 0.05$). Further, AUC was significantly increased in HFD males compared to CD males ($p = 0.006$; Figure 2(c)). This finding further corroborates failure of the clinically relevant glucose-tolerance assay by HFD males detailed above. HFD females did not exhibit an increased AUC compared to CD females ($p = 0.9$; Figure 3(c)), verifying that functional glucose-tolerance was maintained in HFD females. Thus, not only was resting blood glucose elevated, but homeostatic responses to a glucose challenge (glucose-tolerance) were also impaired, only in male but not in female HFD rats.

3.4. HFD Slightly Altered Systemic Insulin Sensitivity in Males but Not Females. The HFD also sex dependently altered systemic blood glucose responses to a bolus injection of insulin, but to a much lesser magnitude than glucose-tolerance testing revealed. Compared to physiological saline injection, blood glucose was significantly reduced in all groups tested 60 min after insulin injection ($F(3, 35) = 5.105$, $p = 0.005$) with diet- and sex-dependent variations in the magnitude, onset, and duration of responses detailed below.

Compared to physiological saline injection (see Figure 2(b)), blood glucose of male CD rats injected with insulin was significantly reduced 60 min after injection ($p = 0.001$), still reduced 90 min after injection ($p = 0.01$), and failed to return to baseline even when tested 120 min ($p = 0.05$) after injection. Compared to comparable responses to physiological saline injections of HFD males (see Figure 2(b)), blood glucose of male HFD rats injected with insulin was significantly reduced 60 min ($p = 0.01$) and 90 min ($p = 0.01$) after injection, but returned to baseline within 120 min after injection ($p = 0.1$), that is, faster than controls.

Compared to physiological saline injections (see Figure 3(b)), blood glucose of female CD rats injected with insulin was significantly reduced 15 min after injection ($p = 0.001$), continued to decline 30 ($p = 0.001$), 60 ($p = 0.001$), and 90 min ($p = 0.001$) after injection, and failed to return to baseline even 120 min ($p = 0.001$) after injection. Compared to the responses of HFD females to physiological saline injections (see Figure 3(b)), blood glucose of female HFD rats injected with insulin was also significantly reduced 15 min after injection ($p = 0.001$), remained reduced 30 ($p = 0.01$) and 60 min after injection ($p = 0.001$), and failed to return to baseline when tested 90 ($p = 0.01$) or 120 min ($p = 0.05$) after injection. Insulin more rapidly depleted circulating glucose in female compared to male rats, and

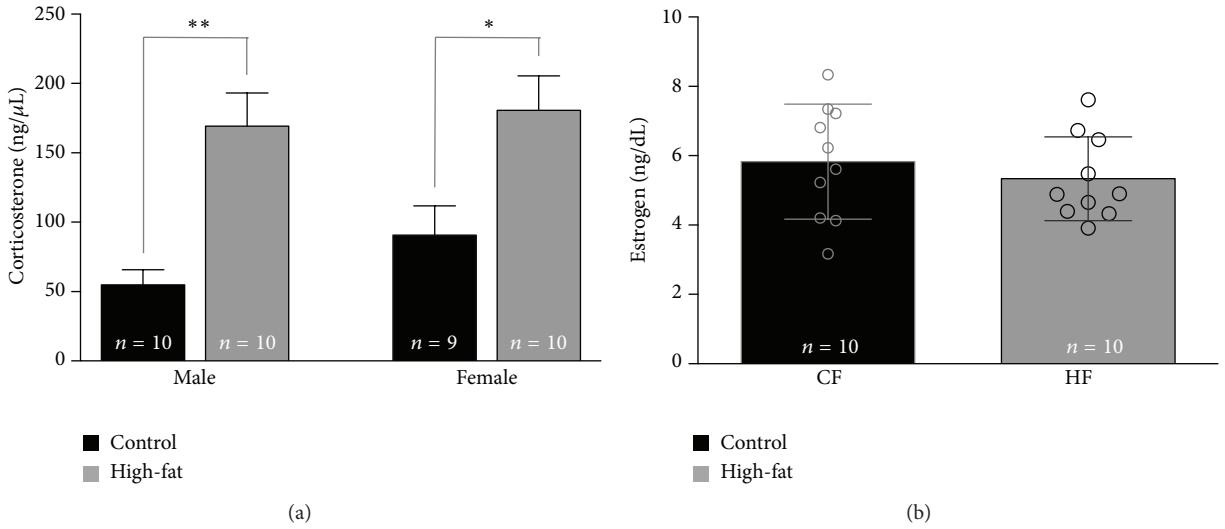


Figure 4: HFD selectively altered circulating corticosterone but not estradiol. The HFD significantly increased circulating corticosterone (a) in both males and females ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Circulating estradiol levels (b), often asserted to be an experimental confound precluding use of females in physiological and behavioral studies, were not significantly altered in females fed the HFD compared to those receiving the CD.

recovery of circulating glucose (return to baseline) was slower in females than in males.

Integrated area above the curve (AAC, a measure of total blood glucose reduction across the insulin-tolerance test interval, compared to the equivalent group's saline-injection curves) was not significantly different between groups tested. AAC was not significantly greater in CD females compared to CD males ($p = 0.1$), nor was it significantly greater in HFD females compared to HFD males ($p = 0.2$). There were no significant differences in AAC comparing CD rats to HFD rats, either in male ($p = 0.6$, Figure 2(d)) or female ($p = 0.9$, Figure 3(d)) cohorts. Although ITT is not used clinically in diagnosis of type 2 diabetes, a maintained ability to respond to the hypoglycemia induced by insulin in both HFD and CD rats indicates that the feedback loop integrating the hippocampus to hypothalamus to pituitary to adrenal cortex (HPA axis) remained functionally intact [17] in all groups tested. Systemic changes in glucose regulation in the HFD groups (in particular, the dysregulation exhibited by HFD males) cannot readily be attributed to insulin intolerance in these young rats. Given the classic diabetic signs of elevated basal fasting glucose in the HFD males, it is likely that these male rats responded, like many type 2 diabetics early in their disease progression, by increasing release of insulin in an attempt to regulate their blood glucose. Preliminary data (not shown) from our laboratory supports this hypothesis and makes assessment of systemic insulin-resistance a complex multifactor issue.

3.5. HFD Increased Circulating Corticosterone in Males and Females. Resting circulating concentrations of serum corticosterone, a major hormone regulating glucose utilization [18], differed significantly between diet treatments (F (3, 34) = 8.15, $p = 0.0003$; see Figure 4(a)), but not between sexes in well-handled rats. Serum corticosterone concentrations were not significantly different in CD fed males compared to females ($p = 0.6$). Corticosterone concentrations were significantly elevated in HFD fed males compared to control fed males ($p = 0.003$) and in HFD fed females compared to control fed females ($p = 0.03$). The high-fat diet did not impact serum corticosterone in males differently from females ($p = 0.98$).

3.6. HFD Did Not Influence Circulating Estradiol. Circulating levels of estradiol did not differ significantly between CD and HFD females ($p = 0.2$; Figure 4(b)). Previous studies suggest that obesity is influenced by sex hormones; female rats gain less weight compared to males when fed a high-fat diet, but this difference is no longer seen after females undergo ovariectomy [13]. Compensatory estradiol-mediated mechanisms do not seem to account for the other sex-dependent metabolic differences observed here.

3.7. HFD Did Not Elevate Circulating Leptin. Leptin, a satiety hormone released by adipose cells, was unaffected by the HFD. Although larger males exhibited higher concentrations of circulating leptin (Figure 5(a)), when corrected for body weight [19] no statistically significant sex- or diet-dependent differences in circulating leptin were observed in young LE rats (Figure 5(b)).

3.8. HFD Impaired Hippocampal-Dependent Spatial Memory in Both Sexes. Spatial memory, assessed via recognition index in a spatial object recognition task (SOR), was significantly impaired in both male and female rats fed the HFD (F (3, 28) = 10.24, $p = 0.0001$, Figure 7(b)). The recognition index was defined as the amount of time spent exploring the novel (moved location; Figure 6(b)) object relative to the total time

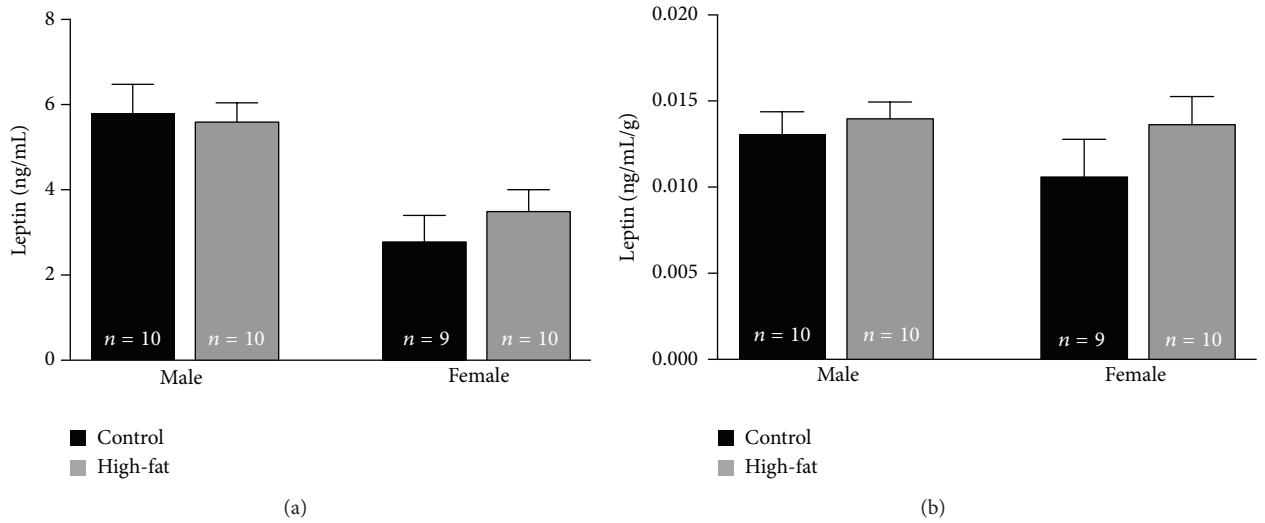
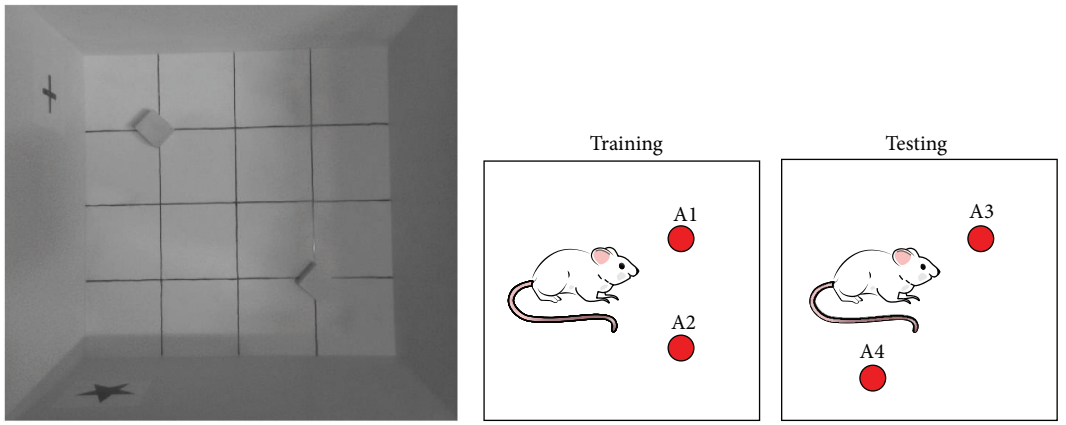


Figure 5: HFD did not significantly alter circulating leptin. Raw data (a) for circulating leptin in males and females appears to show a sex-dependent but not diet-dependent difference in levels of the circulating hormone. However, when leptin concentration is corrected for body weight (b), no significant sex- or diet-dependent differences were observed.



Handling	Habituation	Training	SOR ITI	Testing
7–10 d	3 d	5 min A1 + A2	30 min	5 min A3 + A4^{moved}

(c)

Figure 6: Memory assessed via spatial object recognition (SOR). The task was performed in a 60 × 60 × 60 cm walled open field, with 15 × 15 cm gridlines on the floor and spatial cues positioned on adjacent walls (a). Identical objects were 5 cm solid aluminum cubes. For training, rats were allowed to explore the open field with two objects in defined locations. For testing, rats were again allowed to explore the open field, now with one object in the original location, and one object moved to a novel location (b). The subjects were fully acclimated to handling and to the open field prior to training and testing (c).

spent exploring both objects [RI = $T_N/(T_N + T_F)$] and was used as the primary measure of memory retention [20]. There were no significant differences in total time spent exploring the objects ($p = 0.7$). Additionally, no significant differences were found in other measures of exploration (total line crossings, $p = 0.2$) or of anxiety (center line crossings, $p = 0.2$; time in center, $p = 0.3$), indicating that the memory impairment cannot be attributed to disparities in motor activity or other performance variables between groups (Figure 7(a)). Prior studies have documented HFD-dependent learning and memory impairments in male rats performing hippocampal-dependent tasks [21–23]. McNay et

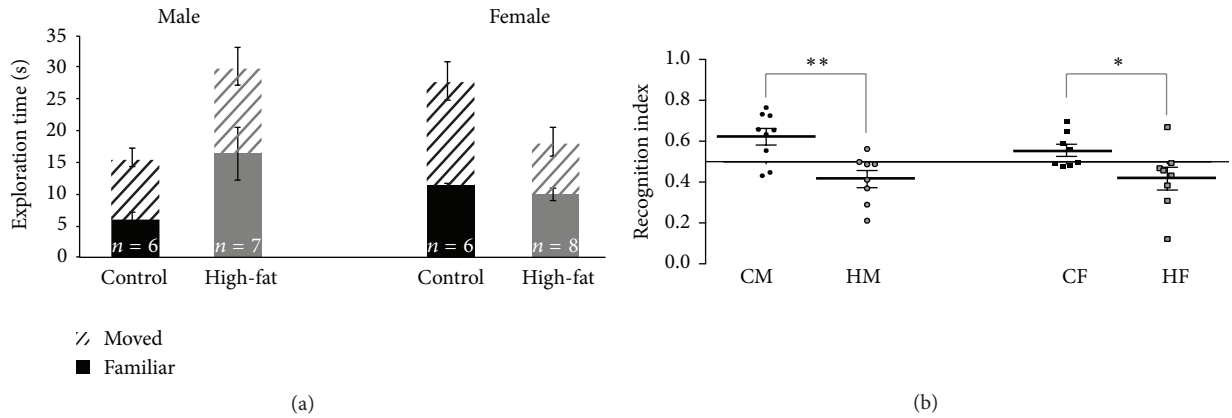


FIGURE 7: HFD impairs spatial memory independent of sex. No sex- or diet-dependent differences were observed in total object exploration (a), total line crosses, centerline crosses, or time spent in the center (data not shown). However, HFD effects were reported in recognition index (b), the comparison of time exploring object in novel location to total time exploring objects ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

al. [2] found spatial memory impairments in male Sprague-Dawley rats with HFD induced obesity (defined as the top tertile by weight gain within their cohort), but no impairment in HFD-resistant rats (defined as the bottom tertile by weight gain). In our hands, there was relatively little variability in distribution of body weights within our diet groups, for both males and females (note the small variance across different ages in Figure 1(a)). While our findings for obese males are concordant with those of McNay et al. [2], the cognitive deficits shown here in HFD females were not accompanied by obesity, by major systemic metabolic changes indicative of onset of type 2 diabetes, nor by peripheral glucose dysregulation. Indeed, the results of the current study strongly suggest that young females ingesting a high-fat diet may be at high cognitive risk, since they may remain largely asymptomatic on systemic measures likely to be clinically assessed (i.e., measures associated with type 2 diabetes: BMI, fasted blood glucose, and glucose-tolerance testing). Our findings highlight an imperative for more research into sex differences, specifically those relating systemic and neural plasticity mechanisms in metabolic disorders, and should be extended across the lifespan.

4. Conclusions

In our rat model, significant weight gain (obesity) was readily induced in male but not in female LE rats ingesting a high-fat diet for approximately 12 weeks (Figure 1(a)) compared to littermate controls. Additionally, diagnostic criteria for type 2 diabetes, including elevated fasting blood glucose (Figure 1(b)) and an impaired glucose-tolerance test (Figures 2(a) and 2(c) [14]), were met by young male but not female HFD rats.

It is important to note that obesity in this and in a large majority of published studies is defined as an overall increase in total body weight [24] compared to controls, in this case induced by obligate consumption of the HFD compared to littermates on standard diet. Systemic measures further validated our model of obesity and metabolic dysregulation in males but not in females, mandating further study to explain these sex-dependent differences. Systematic assessment of peripheral or abdominal body fat is a complex issue [25]. While a previous study of the effects of HFD on cognitive and neural function in middle-aged male Fisher-344 rats found several significant diet-induced increases in different body fat stores and a weak correlation ($r = 0.3$) between measures of systemic lipids and memory measures [24], no significant diet-induced memory impairments were observed. Future work in our and other labs will continue to probe potential lipid-related links.

Our investigation of sex-dependent effects of ingestion of a high-fat diet on young adult rats examined multiple systemic metabolic markers, including these diagnostic of diabetes, and found that despite sex-differences in a variety of these markers, memory performance was equally and significantly impaired on a hippocampal-dependent spatial object recognition task in both male and female rats fed the HFD (Figure 7(b)). Ongoing studies in our laboratory have assessed diet- and sex-dependent changes in hippocampal function which will be reported separately, and diet-induced changes in other brain regions with significant cognitive roles (including neocortex and basolateral amygdala) remain areas of interest. While diet-induced memory impairments have been consistently linked with systemic metabolic impairments when testing has been carried out exclusively in male rodents and other model systems, our divergent findings highlight and reemphasize the need for inclusion of female subjects and direct and systematic comparison with data from males in future studies.

To our knowledge, these are the first findings of sex-dependent dietary changes in systemic glucose regulation, along with sex-independent impairment of hippocampal-mediated cognitive performance. No compensatory changes in estradiol (Figure 4(b)) nor in leptin (Figure 5(b)) were found in comparisons of HFD females to CD females, so additional signals remain to be explored to account for the lack of other systemic changes in glucose regulation

in HFD females. Of the systemic variables assessed, corticosterone alone was significantly elevated in both HFD males and females (Figure 4(a)) and could potentially impact memory performance. Male rats subjected to chronic stress with enhanced circulating corticosteroids fail to remember platform location in a spatial Morris water maze task [26]. However, studies assessing chronic stress (and subsequent corticosterone increase) in females report enhancements in spatial memory performance [27]. While elevations in corticosterone could account for memory impairments seen in male HFD rats, it would not explain the impairment seen in HFD females and require further study.

Additional systemic metabolic markers, as well as an extensive range of signaling pathways within the central nervous system, also remain to be addressed. As noted, other studies in our laboratory have actively explored central effects of the HFD on intrinsic excitability, insulin-sensitivity, and glucose- and insulin-signaling pathways in hippocampal CA1 pyramidal (excitatory output) neurons, as well as on performance on other hippocampal-dependent memory tasks, and continue to strengthen the case for the need for comparative studies in both males and females under the same conditions. Since hippocampal neurons are reciprocally connected with numerous neocortical regions and cortical neurons also express abundant IRs (and insulin-resistance has been reported in cortical regions of Alzheimer's patients) [28, 29], the effects of HFD on hippocampus, neocortex, and other brain regions will continue to be assessed in future studies of HFD-related cognitive decline.

While the consequences of sex differences in development and impact of type 2 diabetes can be profound, comparative metabolic studies in young and young adult model systems are rare, despite alarming human population trends in youth [1]. Obese women with type 2 diabetes have a higher occurrence of cognitive decline than men [30] as they age. Obese women are twice as likely to have dementia as women of normal weight, while obese men are at no greater risk than normal weight men [31]. Long-term consequences of obesity, glucose dysregulation, and consequent neuronal dysfunction must be studied in parallel in males and females, since a one-size-fits-all approach cannot adequately detail or identify all relevant issues.

Conflict of Interests

The authors declare no competing financial interests.

Acknowledgments

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Research Article

Repeated Three-Hour Maternal Separation Induces Depression-Like Behavior and Affects the Expression of Hippocampal Plasticity-Related Proteins in C57BL/6N Mice

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Adverse early life experiences can negatively affect behaviors later in life. Maternal separation (MS) has been extensively investigated in animal models in the adult phase of MS. The study aimed to explore the mechanism by which MS negatively affects C57BL/6N mice, especially the effects caused by MS in the early phase. Early life adversity especially can alter plasticity functions. To determine whether adverse early life experiences induce changes in plasticity in the brain hippocampus, we established an MS paradigm. In this research, the mice were treated with mild (15 min, MS15) or prolonged (180 min, MS180) maternal separation from postnatal day 2 to postnatal day 21. The mice underwent a forced swimming test, a tail suspension test, and an open field test, respectively. Afterward, the mice were sacrificed on postnatal day 31 to determine the effects of MS on early life stages. Results implied that MS induces depression-like behavior and the effects may be mediated partly by interfering with the hippocampal GSK-3β-CREB signaling pathway and by reducing the levels of some plasticity-related proteins.

1. Introduction

In mammals, adverse life events that occur in early neuronal development can change normal brain growth and stress vulnerability in adulthood [1]. Acute or chronic stressful periods, particularly during childhood and adolescence, induce the onset of emotional and affective disorders, such as depression and anxiety [2]. The time and duration of any stressful experience that occurs in the neonatal or adolescent period are possibly necessary to promote proper neuronal organization; these parameters can also exacerbate the vulnerability to long-term behavioral changes [3]. Our study generally aimed to assess the mechanism by which the association between neonatal and adolescent stressful experiences may influence stress responsiveness and brain plasticity in C57BL/6N mice.

We induced MS in male mice, an established model of adverse early life experiences. Considering depression, researchers hypothesized that structural plasticity and neurotrophic factors are necessary to mediate behavioral responses to MS. For example, neurofilament light chain (NF-L) is a reliable marker of structural plasticity; this marker indicates neuronal impairment at a molecular level. NF-L is also a subunit of neurofilaments (NFs). NFs are neuron-specific cytoskeletal filaments found in most mature neurons. NFs provide structural support for neurons and their synapses; NFs also maintain and control neuronal cytoskeletal plasticity by regulating neurite outgrowth, axonal caliber, and axonal transport [4]. In addition to NF-L, brain-derived neurotrophic factor (BDNF) is a key regulator of neuronal plasticity. BDNF strongly affects synaptogenesis, spine formation [5], neuronal survival [6], long-term potentiation, neuronal excitability [7], and adult hippocampal neurogenesis [8]. The transcription of several genes, such as BDNF, is stimulated by activating the phosphorylation of cAMP response element-binding

protein (CREB) (Ser133) [9]. CREB is regarded as a key nucleoprotein related to depression and antidepressant treatments [10].

The transduction pathways and upstream signaling molecules of CREB-BDNF are complex; among these molecules, glycogen synthase kinase-3 (GSK-3) is a newly reported inhibitory signaling molecule [11–13]. This kinase was originally identified as a key enzyme of glucose metabolism. GSK-3 is considered as a broadly influential enzyme that affects a diverse range of biological functions because this enzyme regulates a large group of transcription factors and transcriptional modulators [14]. GSK-3 can be directly inhibited by the mood stabilizer lithium [15]; this result suggests that GSK-3 may be associated with the pathophysiology of mood disorders. Furthermore, GSK-3 exists in two closely related isoforms, namely, GSK-3α and GSK-3β. The constitutively active GSK-3β is an important regulatory protein involved in many neuroplasticity-associated intracellular signaling pathways [16]. In our study, the MS-induced depression-like behavior and some hippocampal plasticity-related proteins were observed in male C57BL/6N mice; the effects on the MS model were then investigated. The underlying mechanism was also determined on the basis of the GSK-3β-CREB signaling pathway.

2. Materials and Methods

2.1. Experimental Animals. The pregnant C57BL/6N mice were obtained from the Laboratory Animal Center of Nanjing University of Chinese Medical. The mice were housed in groups of four in home cages made of Plexiglas (35 cm × 15 cm × 10 cm) with sawdust bedding. The animals were maintained under a standard dark-light cycle (lights on between 6:00 and 18:00) at room temperature of 22 ± 2°C. The mice had free access to food and water. Prior to the experiments, mice were habituated to daily handling during the week after delivery. All animals treatments were in accordance with the Guidelines of Accommodation and Care of Animals formulated by the Chinese Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes. All efforts were made to minimize animal suffering and reduce the number of animals used for experiments.

2.2. Experimental Design. The mouse was born at the first day postpartum (PD1), respectively, from PD2 to PD21 of MS (MS took place when the mouse was moved to a single cage); the mice were moved to the incubator (30 to 32°C). MS180 consisted of daily separation of litters from their dams and sires for 3 h (09:00–12:00 h), while MS15 involved daily brief separations of 15 min (09:00–09:15 h), as shown in Figure 1.

2.3. Behavioral Tests. Behavioral tests were performed in JLBehv-FSG-4 sound insulation boxes with the DigBehav animal behavior video analysis system (Shanghai Jiliang Software Technology Co., Ltd., Shanghai, China). DigBehav can automatically record and analyze animal movements to provide total immobility times during the FST and TST. Depression-like behavior was inferred from the increasing time spent immobile during these tests. The FST method was similar to that described by Porsolt et al. [17]. Considering the younger ages of our experimental mice, shorter body length, and the optimization of our preexperiment, the mice were placed individually in 10 cm deep water at ambient temperature (25 ± 1°C) in a 2000 mL glass beakers and were allowed to swim for 5 minutes. Plus, the strength of our mice was weaker compared with adult ones. Taking the above into account, the adaptation time was shortened to 1 minute. The duration of immobility was recorded during the last 4 min of the test. The TST method was similar to that described by Steru et al. [18]. After the FST, the mice were allowed to rest for 24 h. Each mouse was then suspended on the edge of a shelf at 58 cm above the bottom of the sound insulation box, using adhesive tape placed approximately 1 cm from the tip of the tail. The animals were allowed to hang for 6 min, and the duration of immobility was recorded during the last 4 min of the test.

2.4. Real-Time PCR Analysis. Total RNA from bilateral hippocampal tissue was extracted using Trizol reagent (cwbio, cw0580). cDNA was synthesized with 2 μg of total RNA using the RevertAid Transcript First-Strand cDNA Synthesis Kit (Fermentas, K1622). Quantitative real-time PCR was performed using the SYBR Green Master Mix (Fermentas, K0222) in the StepOne Real-Time PCR System (ABI, USA). The sequences of primers were BDNF forward: 5′-GGTCACAGCGGCAGATAAAAAGAC-3′, reverse: 5′-TTGGGTAGTTCGGCATTGCGAG-3′; NF-L forward: 5′-GTTCAAGAGCCGCTTCACCG-3′, reverse: 5′-CCAGGGTCTTAGCCTTGAGCAG-3′; GAPDH forward: 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, reverse: 5′-CATGTGGGCCATGAGGTCCACCAC-3′. The following thermal cycling conditions were used: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and then annealing and extension, both at 60°C for 1 min. The amplification of only a single sequence was verified by the dissociation curve of each reaction. All experiments were performed in triplicate, and the average threshold cycle (Ct) value was the extreme Ct value of the sample. The mRNA expression of GFAP was calculated relative to the house keeping gene GFAP using the $2^{-\Delta Ct}$ method, $\Delta Ct = Ct_{(\text{the target gene})} - Ct_{(GFAP)}$.

2.5. Western Blot Analysis. Bilateral hippocampal tissue samples were homogenized at 4°C in 0.5 mL of lysis buffer containing 50 mM Tris-HCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet-P40 (NP-40, Sigma), 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (Sigma), 1 mM NaF, 1 mM Na_3VO_4, 1 μgmL^{-1} aprotinin (Sigma), and 1 μgmL^{-1} leupeptin (Sigma) (pH 7.5). Aliquots of the clarified homogenized liquid, containing 75 μg of protein, were denatured at 95°C for 5 min in a sample buffer containing 1% SDS, 1% dithiothreitol (Sigma), 10 mM Tris-HCl, 10% glycerol, and 1 mM EDTA (pH 8.0). The sample proteins were then separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad). The primary antibodies used to examine the changes in

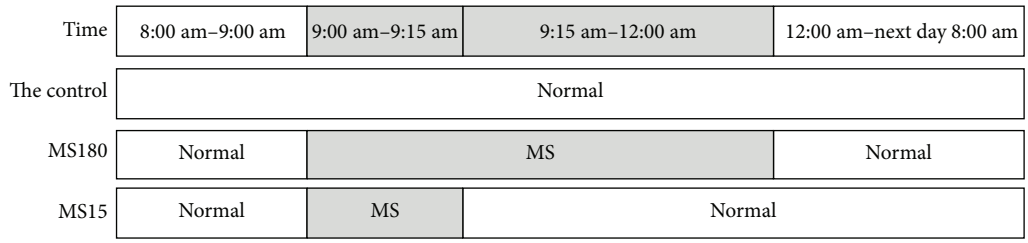

Figure 1: MS180 consisted of daily separation of litters from their dams and sires for 3 h (09:00–12:00 h), while MS15 involved daily brief separations of 15 min (09:00–09:15 h).

Table 1: Offspring weight across development.

Group	PND2	PND7	PND14	PND21	PND28
Control	1.58 ± 0.02	4.67 ± 0.07	7.14 ± 0.11	9.79 ± 0.23	12.34 ± 0.31
MS180	1.59 ± 0.02	4.62 ± 0.08	6.76 ± 0.14*	9.34 ± 0.21**	11.77 ± 0.28**
MS15	1.58 ± 0.03	4.65 ± 0.06	7.11 ± 0.12	9.82 ± 0.20	12.32 ± 0.27

MS180 reduced body weight versus with control. $^{*}P < 0.05$ statistical significance to control and MS15. $^{**}P < 0.01$ significant difference to control and MS15. PND = postnatal day.

protein expression included the rabbit polyclonal anti-BDNF antibody (1 : 200, Abcam, ab6201), the mouse monoclonal anti-NF-L antibody (1 : 500, Invitrogen, 13-0400), the rabbit monoclonal anti-CREB (1 : 1000, Cell Signaling, 9197S), the rabbit monoclonal phospho-CREB (Ser133) (1 : 1000, Cell Signaling, 9198S), the rabbit monoclonal anti-GSK-3β (1 : 1000, Cell Signaling, 9315S), the rabbit monoclonal anti-phospho-GSK-3β (Ser9) (1 : 1000, Cell Signaling, 9323S), and the mouse monoclonal anti-β-actin (1 : 2000, Sigma, A1978). The secondary antibodies included the horseradish peroxidase conjugated goat anti-mouse IgG (1 : 4000, GenScript) and the goat anti-rabbit IgG (1 : 4000, GenScript). Immunoblotting was detected by enhanced chemiluminescence (Bio-Rad XRS+) and analyzed using ImageLab 5.0. The values of the BDNF, NF-L, CREB, and GSK-3β levels were normalized against the amount of β-actin obtained from the same sample. The phospho-GSK-3β/GSK-3β and phospho-CREB/CREB were calculated to reflect the activity of GSK-3β and CREB. Three protein samples per animal were examined for each target protein.

2.6. Statistical Analysis. Data were expressed as the mean ± SEM for the indicated number of experiments and analyzed using the Statistical Package for Social Sciences computer program (version 20.0). The statistical significance of the results was determined using one-way ANOVA, followed by Tukey's post hoc tests. The significance level was set at $P \leq 0.05$ for all statistical comparisons.

3. Results

3.1. Effects of Maternal Separation on Body Weight. Offspring weight was assessed on PND2, PND7, PND14, PND21, and PND28. A difference in weight was observed on PND14 ($F_{2,27} = 4.07$, $P < 0.05$), PND21 ($F_{2,27} = 5.74$, $P < 0.01$), and PND28 ($F_{2,27} = 6.11$, $P < 0.01$), where in MS180, the body weight were lighter than Control and MS15 since PND14 to PND28 (Table 1).

3.2. Effects of Maternal Separation on Immobility Time in the Mouse TST. In TST ($F_{2,27} = 3.69$, $P < 0.05$), the immobility times differed significantly among the groups. Multiple comparison tests revealed that MS180 induced a significant increase in immobility time compared with the control group and the MS15 group in the TST ($P < 0.01$). MS15 and the control group had no significant difference ($P > 0.05$) (Figure 2).

3.3. Effects of Maternal Separation on Immobility Time in the Mouse FST. In FST ($F_{2,27} = 4.52$, $P < 0.05$), the immobility times differed significantly among the groups. Multiple comparison tests revealed that MS180 induced a significant increase in immobility time compared with the normal group and MS15 in the FST ($P < 0.01$). MS15 and the control group had no significant difference ($P > 0.05$) (Figure 3).

3.4. Effects of Maternal Separation on Distance in the Mouse Open Field Test. In open field test ($F_{2,27} = 0.409$, $P > 0.05$), the results showed no statistical significance between groups (Figure 4).

3.5. Maternal Separation Reduced the Hippocampal mRNA Levels of BDNF and NF-L in C57BL/6N Mice. The relative target gene mRNA levels of the groups are shown in Figure 5. The ANOVA tests showed a significant effect of the groups in the hippocampal mRNA level of BDNF ($F_{2,27} = 7.94$, $P < 0.01$) and NF-L ($F_{2,27} = 8.29$, $P < 0.01$). Post hoc comparisons revealed that the MS180 significantly decreased the hippocampal mRNA levels of BDNF and NF-L compared with the control group and MS15 ($P < 0.01$).

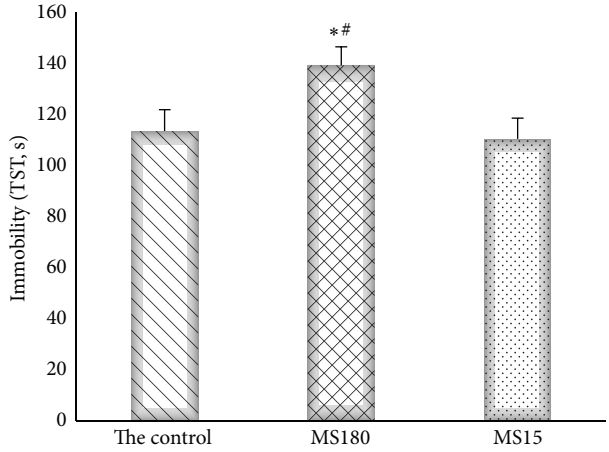

Figure 2: The tail suspension immobility time in animal testing. $^{*}P < 0.01$ versus the control group, $^{\#}P < 0.01$ versus the MS15 group.

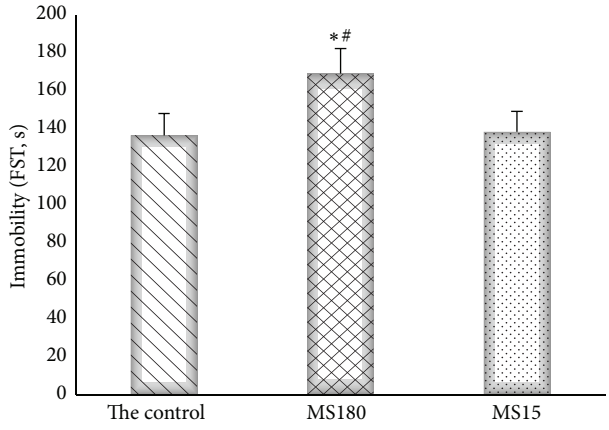

Figure 3: The forced swimming test of static time. $^{*}P < 0.01$ versus the control group, $^{\#}P < 0.01$ versus the MS15 group.

3.6. Maternal Separation Reduced the Hippocampal Protein Levels of GSK-3β Inhibitory Phosphorylation, CREB Activation, BDNF, and NF-L. Figure 6 shows that the hippocampal protein level of GSK-3β was significantly higher in the MS180 group than in the control group and the MS15 group ($F_{2,27} = 5.77$, $P < 0.01$). The ratios of phospho-GSK-3β (Ser9) and GSK-3β were significantly different among the groups ($F_{2,27} = 7.13$, $P < 0.01$). By contrast, the expression of the hippocampal CREB protein was not significantly different among the groups ($F_{2,27} = 1.46$, $P > 0.05$). Post hoc comparisons revealed that the ratio of phospho-CREB (Ser133) was lower in the MS180 group than in the control group and the MS15 group ($F_{2,27} = 11.07$, $P < 0.01$). The hippocampal protein levels of BDNF and NF-L in the MS180 group were significantly lower than those in the control group and the MS15 group ($F_{2,27} = 8.53$, $P < 0.01$).

4. Discussion

Previous studies revealed that MS induced acutely or subacutely to normal mice elicits depression-like effects [19–22].

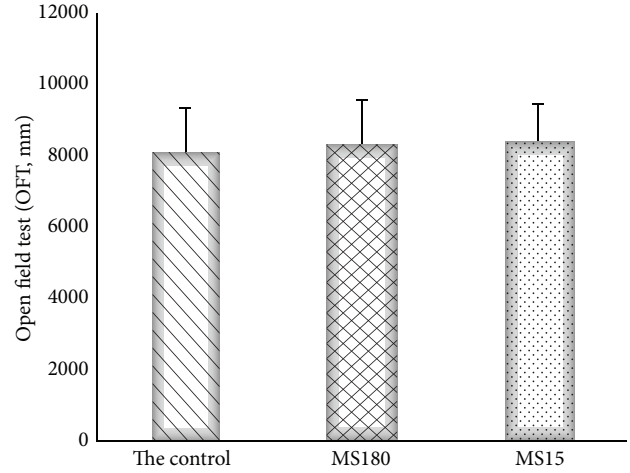

Figure 4: The open field test on shifting distance. $P > 0.05$ versus the control group and the MS15 group.

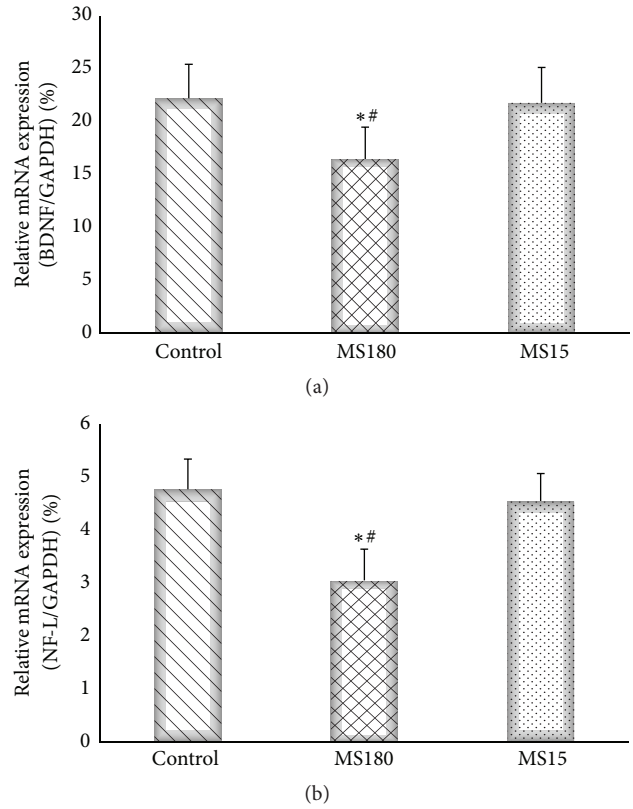

Figure 5: Effects of MS on the hippocampal mRNA levels of BDNF (a) and NF-L (b) in the mouse model. $^{*}P < 0.01$ versus the control group and $^{\#}P < 0.01$ versus the MS15 group.

However, previous studies focused on the changes in the adult phase of MS. Few studies have demonstrated the effects of MS on the changes in the early phase of mice, although the early life stages of mice are a key period in the development of the nervous system. Hence, further studies should be conducted to explore the effects of MS on early life stages.

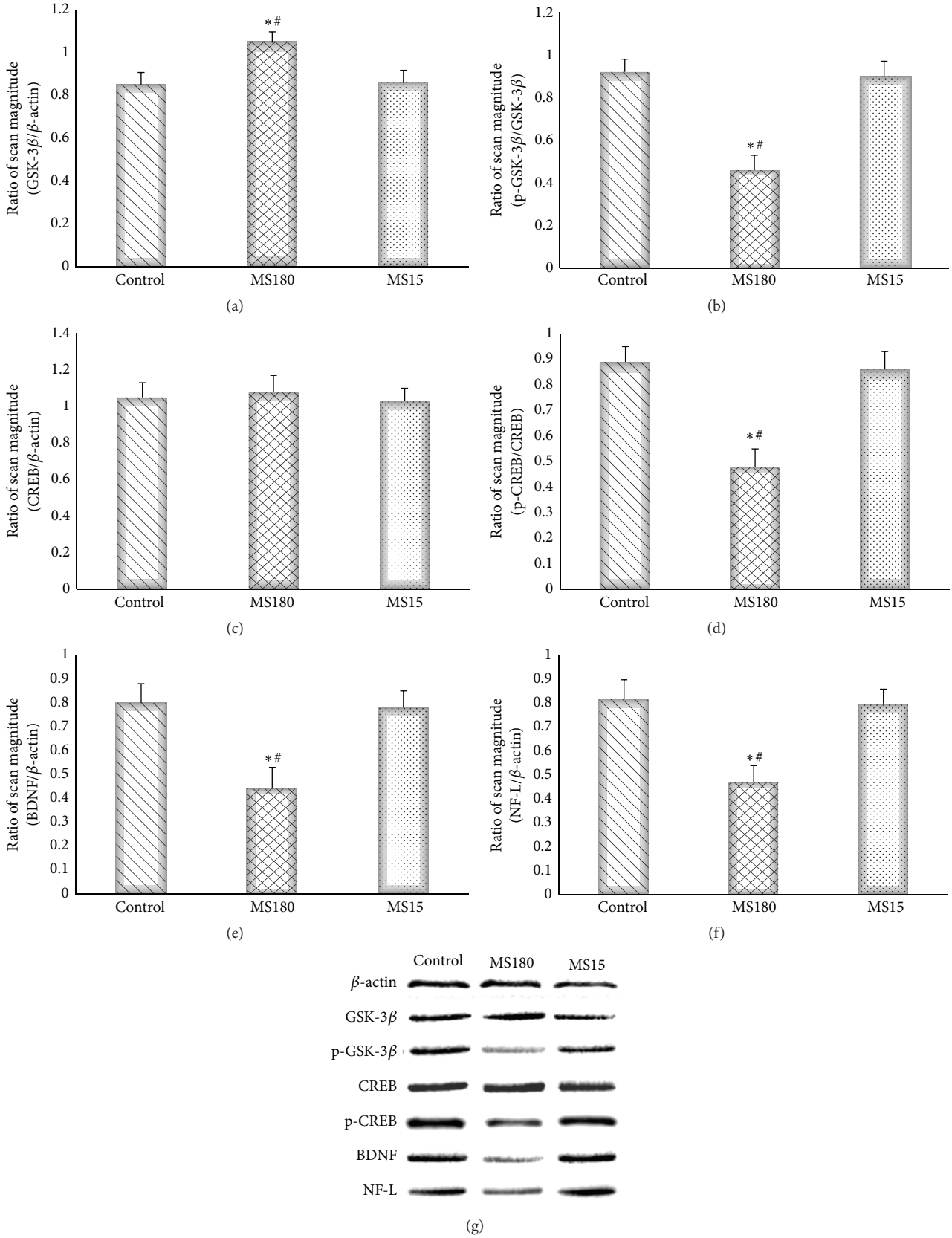

Figure 6: Hippocampal GSK-3β, p-GSK-3β, CREB, p-CREB, BDNF, and NF-L protein levels in the MS-induced mouse model were determined by Western blot analysis. The values of GSK-3β (a), CREB (c), BDNF (e), and NF-L (f) levels were normalized against the amount of β-actin, while the values of p-GSK-3β (Ser9) (b) and p-CREB (Ser133) (d) were normalized against the amount of GSK-3β and CREB, respectively. $^{*}P < 0.01$ versus the control group, $^{\#}P < 0.01$ versus the MS15 group.

Our study demonstrated that MS180 significantly reduced the immobility time in FST and/or TST. Depression affects spontaneous locomotor activity [23]; as such, the reduced immobility time may account for the MS-induced depression. The behavioral results of our study are consistent with those described in previous studies, which examined the effects of MS. However, the mice in our study were monitored on postnatal day 31, not on the usual age of more than eight weeks.

Consistent with previous findings, our results confirmed that MS180 induced depression-like behavior and hippocampal impairment in mice. These results were based on the increased immobility time in the behavioral tests and the reduced expression of BDNF and NF-L in the hippocampus. Moreover, MS180 evidently induced these depression-like effects; by contrast, MS15 did not significantly affect the BDNF and NF-L protein levels and the behavioral test results. Considering that MS15 did not significantly reduce the BDNF or NF-L expression in the hippocampus of normal mice, we concluded that MS induced the depression-like behavior as a result of the decreased expression of these plasticity-related proteins; thus, neuroplasticity may be inhibited.

Neuroplasticity-related signaling pathways may be involved in the pathophysiology and mechanisms of depression [24, 25]. In our study, this issue was addressed by investigating the involvement of the CREB-BDNF signaling pathway in the hippocampus. The downregulation of the hippocampal BDNF expression has been demonstrated in various animal depression models and in depressed patients; the chronic treatment of several classes of antidepressants increases the BDNF expression [26]. As an upstream transcriptional activator of BDNF, the hippocampal CREB expression is decreased among experimental animals exposed to specific stressors [27, 28]. A decrease in the CREB expression has also been observed in depressed patients [29, 30]. Our results showed that MS180 normalized the downregulated hippocampal mRNA and protein levels of BDNF; MS180 also reduced the activation of CREB in the C57BL/6N mouse model. This result further confirmed the depression-like effects of long-term MS; this result also suggested that the depression-like effects of MS180 may be due to the inhibition of CREB-BDNF in the hippocampus. Furthermore, MS15 did not induce any significant depression activity in the behavioral tests. MS15 could not also affect the mRNA expression of BDNF and the protein expression of phospho-CREB (Ser133) in the hippocampus. Therefore, short-term MS did not induce depression; by contrast, long-term MS could cause depression.

Previous studies on the effects of adverse early life experiences on the CNS focused on BDNF because of the unique role of this molecule in the CNS. However, studies have rarely investigated the mechanism by which MS influences the upstream signaling pathway of BDNF. Our study focused on GSK-3β, another upstream signaling molecule of CREB-BDNF, because GSK-3β is involved in various signaling systems [14] and is possibly associated with mood disorders [11].

GSK-3β and phospho-GSK-3β (Ser9) were also investigated in this study. Our results showed that MS180 increased the GSK-3β expression and reduced its inhibitory phosphorylation. Consistent with previous findings on depressed rats and patients [31, 32], our results further confirmed that insufficient GSK-3β inhibition is a risk factor of depression. In our study, MS180 significantly downregulated the inhibitory phosphorylation of GSK-3β in our model; thus, MS180 may activate GSK-3β. To the best of our knowledge, this study is the first to investigate the effect of MS on the early life phase of mice and to examine the hippocampal GSK-3β level and activity in this mouse model. This study is also the first to reveal the effects of MS on GSK-3β in this model. GSK-3β participates in several intracellular signaling pathways involved in neuroprotection [16]. Our results suggested that the GSK-3β-CREB signaling pathway may contribute to the decreased expression of some plasticity-related proteins in the hippocampus; this pathway may also induce depression-like behaviors. Moreover, the MS-induced activity of the GSK-3β-CREB signaling pathway is a possible mechanism of depression.

5. Conclusion

The structural plasticity of the hippocampus is critical for adverse early life experiences. In the MS model, MS180 induced depression-like behaviors and decreased the expression of some plasticity-related proteins. MS180 also inhibited the CREB-BDNF signaling pathway in the hippocampus. Furthermore, MS180 decreased the inhibitory phosphorylation of GSK-3β; as a result, the CREB-BDNF signaling pathway was inhibited. Thus, this inhibition may account for the depression-like activity of MS180.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Yaoyao Bian, Lili Yang, and Zhongli Wang contributed equally to the paper.

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