Amyloid β lowering and cognition enhancing effects of ghrelin receptor analog [D-Lys (3)] GHRP-6 in rat model of obesity

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Obesity arising due to the dietary and lifestyle changes is fast reaching epidemic proportions all over the world. There is increasing evidence that the incidence of Alzheimer disease (AD) is significantly influenced by a cluster of metabolic diseases, including diabetes and obesity. This study was aimed to test the suitability of experimentally-induced obesity in rats as an experimental animal model of AD. We used the procedure of neonatal administration of rats with monosodium L-glutamate (MSG), which generates adult obese animals as our study design and assessed the AD-like changes by measuring amyloid β (1-42) and acetylcholinesterase (AChE) levels in the hippocampal extracts and cognitive impairments by Barnes maze task. Further, we investigated the influence of anti-obesity substance [D-Lys (3)] GHRP-6 on blood glucose, hippocampal Aß, AChE levels and restoration of cognitive deficits. Results revealed that administration of MSG to neonatal rats exhibited increased body mass index and serum glucose levels over the controls. Measurement of markers for AD-like molecular changes i.e. amyloid β (Aß) and AChE levels showed marked elevation in these two parameters in the hippocampus of MSG-treated rats. Assessment of cognitive abilities by Barnes maze revealed spatial disorientation characteristic of AD. Administration of ghrelin receptor analog [D-Lys (3)] GHRP-6 to obese rats resulted in significant restoration of serum cholesterol, glucose, leptin and ghrelin levels to that of control with concomitant reduction in hippocampal Aß and AChE levels. In addition, the treated animals exhibited marked improvement in Barne’s maze task. These findings suggest that MSG-induced obese rats may serve as non-transgenic animal model for AD research. Further, the results indicate the potential of [D-Lys (3)] GHRP-6 as a promising anti-Alzheimer candidate.

Keywords: Amyloid β, Acetylcholinesterase, Alzheimer disease, Monosodium glutamate, Obesity, Spatial memory, Ghrelin receptor analog [D-Lys (3)] GHRP-6

Alzheimer’s disease (AD) is one of the leading causes of dementia in the elderly, with more than 30 million individuals currently affected worldwide. AD is caused by the selective loss/dysfunction of neurons, particularly those expressing nicotinic acetylcholine receptors¹ in specific brain regions, including the neocortex and hippocampus². Spatial learning, working memory and long-term memory impairments are some of the earliest symptoms expressed in AD³. The pathological hallmarks of AD include intracellular neurofibrillary tangles (NFT) consisting of hyperphosphorylated form of the microtubule associated protein tau and senile plaques comprising of the aggregated peptide amyloid β (Aß)⁴. An increase in acetylcholinesterase (AChE), a key enzyme in the cholinergic nervous system, around the senile plaques and NFT is a common feature of AD neuropathology⁵. Aß (1-42) is generated from a larger precursor called amyloid precursor protein (APP) by the proteolytic action of specific cleavage enzymes called ß- and γ-secretases. Mutations in either the Aß precursor protein or processing enzymes account for familial cases of AD (<5%). However, vast majority of AD cases (>95%) are sporadic, significantly influenced by the presence of an apolipoprotein allele E4⁶, dietary lifestyle⁴ and more importantly, the metabolic disorders, such as diabetes⁸ and obesity⁹.

Research into the etiology of AD with animal models mimicking aspects of the disorder has substantially contributed to the advancement of AD research¹⁰. A number of investigations have also identified and explored non-transgenic approaches to animal model systems of AD, which include senescence accelerated mouse strains¹¹, streptozotocin-induced diabetic rats¹²,¹³, guinea pigs¹⁴, beagles¹⁵ and non-human primates¹⁶ as experimental models of AD. A recent study¹⁷ has reported Alzheimer-type pathological changes in morbidly obese elderly individuals. This has prompted us to evaluate the
suitability of experimentally-induced obese rats as an animal model of AD. Towards this, we have used the procedure of neonatal administration of rats with monosodium L-glutamate (MSG), which generates adult obese animals as our study design. We have assessed the AD-like changes by measuring Ab (1-42) and AChE levels in the hippocampal extracts and cognitive impairments by Barnes maze task. Further, we have investigated the influence of anti-obesity substance [D-Lys (3)] GHRP-6 on blood glucose, hippocampal Ab, AChE levels and restoration of cognitive deficits.

Materials and Methods

Materials
Monosodium L-glutamate (MSG), [D-Lys (3)] GHRP-6, phenylmethanesulphonyl fluoride (PMSF), 5, 5-dithiobis-2-nitrobenzoic acid, acetylthiocholine (ATC), glucose oxidase, peroxidase, 4-aminophenazone and bovine serum albumin were purchased from Sigma-Aldrich, Bangalore. ELISA kit for estimation of rat Ab (1-42), leptin and ghrelin were purchased from Uscn Life Science Inc, Wuhan, P.R. China. All other reagents used were of analytical grade and obtained locally.

Animals and experimental protocols
Male Sprague Dawley rat pups were chosen for the study. They were maintained under controlled temperature and light. Neonatal rat pups were injected with MSG (4 mg/g body weight) once daily for 14 consecutive days after birth through intraperitoneal route. Rats of the same age and strain, receiving saline served as control. After 14 days of drug administration, the rats were allowed to grow till the time of sacrifice with ad libitum access to pellet food and water. After 3-, 6-, 9- and 12-months of age, body weight and naso-anal length were recorded.

To one group of MSG treated 12-month-old rats, a single intraperitoneal injection of [D-Lys3]-GHRP 6 (1 mg/Kg body weight) in saline was administered. After 15 days, the animals were sacrificed by cervical dislocation. Approx. 1 ml of blood was collected from each animal through cardiac puncture, serum separated and stored at -20°C until analysis. The brain was quickly removed, hippocampi dissected, weighed and stored frozen at -20°C for further analysis. All procedures were approved by the Institutional Animal Ethics Committee.

Behavioural assessment
Before sacrifice, subjects underwent testing in the Barnes maze task to evaluate the spatial learning and memory. During training phase, each rat underwent 3 successive trials per day for 5 consecutive days. During the trials, rats from each group were randomly assigned to locate the escape tunnel from one of the four predetermined locations to rule out spatial preference. Moderately noxious stimuli, blowing fans and 200W of bright light were used to increase the incentive in finding the escape tunnel. Data represented as the spatial disorientation score defined by the number of wrong holes searched before identifying the escape tunnel.

Measurement of serum parameters
Total cholesterol and serum glucose were estimated by the previously described methods. Serum leptin and ghrelin levels were quantitated using commercially available ELISA kits as per manufacturer’s instructions.

Homogenization of hippocampi and measurement of AChE activity and Ab
The rats were sacrificed by cervical dislocation and the brains were removed. Hippocampi were dissected and homogenized in ice-cold 50 mM Tris-HCl buffer, pH 7.4 containing 150 mM NaCl, 2 mM EDTA, 1 mM PMSF and 0.5% Triton X-100, followed by sonication (10 s × 2 cycles). Homogenates were then centrifuged at 13 000 × g for 20 min. The clear extracts obtained were used for estimation of total protein content by Lowry’s method using bovine serum albumin as standard. An aliquot of the extract was used for quantitative measurement of Ab by sandwich ELISA method as per manufacturer’s instructions.

The esterase activity was measured in the hippocampal extract by using an artificial substrate acetylthiocholine (ATC). Thiocholine released because of the cleavage of ATC by AChE was allowed to react with the -SH reagent 5, 5-dithiobis-2-nitrobenzoic acid, which was reduced to thionitrobenzoic acid, a yellow coloured anion with an absorption maxima at 412 nm. All samples were run in triplicate and the enzyme activity was expressed as µ mol ATC/h/mg protein.

Statistical analysis
The data were expressed as mean ± S. D. and differences between groups were analyzed by ANOVA, followed by Tukey’s multiple comparisons using
Obesity, diabetes and aging, the characteristics of the human population are on the rise across the globe. It has long been established that aging is the major risk factor for AD, and it is becoming increasingly evident that obesity is emerging as another risk factor. Mid-life obesity, assessed by both body mass index (BMI) and skin fold thickness have consistently shown a strong and independent association with an increased risk of dementia and AD. Alzheimer-type neuropathological changes have been noted in morbidly obese elderly individuals (>65 yrs of age) without clinical history of cognitive impairment, approaching those seen in AD for some patients. These observations prompted us to validate the use of experimentally-induced obese rats as a non-transgenic animal model of AD.

As a first step, we generated the obese rats in our laboratory (Fig. 1A) by following the demonstrated procedures. It was evident from the data presented in Table 1 that MSG-induced obese rats showed significant weight increase, increased nasoanal length, and increased BMI compared to control rats. These changes were accompanied by increased serum glucose and cholesterol levels, and reduced spatial disorientation scores.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3-months-old rats</th>
<th>6-months-old rats</th>
<th>9-months-old rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>227.7 ± 2.52</td>
<td>263.3 ± 49.3 n.s.</td>
<td>269 ± 10.54</td>
</tr>
<tr>
<td>Nasoanal length (cm)</td>
<td>21.67 ± 0.58</td>
<td>17.0 ± 1.0**</td>
<td>23.67 ± 0.58</td>
</tr>
<tr>
<td>BMI (n.s.)</td>
<td>0.485 ± 0.03</td>
<td>0.894 ± 0.09**</td>
<td>0.48 ± 0.013</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>118.3 ± 7.6</td>
<td>131.7 ± 2.9*</td>
<td>111.7 ± 5.8</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dl)</td>
<td>165.3 ± 13.6</td>
<td>277.7 ± 87.5 n.s.</td>
<td>170.3 ± 25</td>
</tr>
</tbody>
</table>

AD associated changes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3-months-old rats</th>
<th>6-months-old rats</th>
<th>9-months-old rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight of hippocampus (mg)</td>
<td>86.7 ± 10.7</td>
<td>54 ± 6.0**</td>
<td>93.3 ± 7.0</td>
</tr>
<tr>
<td>Aß (pg/mg protein)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>AChE activity (µ mol substrate/h/mg protein)</td>
<td>1.0 ± 0.42</td>
<td>5.4 ± 1.2*</td>
<td>1.0 ± 0.28</td>
</tr>
<tr>
<td>Spatial disorientation score</td>
<td>4.5 ± 5.7</td>
<td>6.9 ± 3.0 n.s.</td>
<td>6.0 ± 4.0</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.001 and ***P<0.0001 compared to age-matched controls.

BMI calculated as a ratio of weight of the animal (g) and the square of the nasoanal length (cm). n.s., not significant. N.D., not detectable.
increased body weight, shortened height, higher BMI, significantly elevated levels of glucose and cholesterol in the serum, compared to saline-treated control group. In addition, administration of MSG resulted in significant loss in the hippocampal weight (Table 1), with overall reduction in the brain size (Fig. 1B). These results were in agreement with the recent findings that obesity, as measured by BMI, is associated with lower hippocampal volumes in mildly affected AD patients and that these animals could be considered useful as experimental model for developing a high rate of diabetic obesity, as observed with MSG-induced obesity studies.

After establishing obesity in the adult rats, we next measured the amount of Aβ and the enzyme activity levels of AChE in the hippocampal extracts prepared from the saline control and MSG-treated rats. The results (Table 1) clearly demonstrated significant increment in the amount of Aβ in the obese rats over the control group at 6- and 9-months of age. In parallel, a significant increase in the AChE activity levels was observed in the MSG-treated group. AChE has consistently been shown to increase in regions around amyloid plaques at all stages of AD, including some of the earlier stages. Studies by Rees et al. have suggested the direct interaction between AChE and Aβ and that AChE may play a role in pathogenesis of AD. Using different experimental conditions, Garcia-Ayllon et al. have confirmed that Aβ triggers an increase in AChE, which can in turn affect Aβ production by modulating the levels of the γ-secretase catalytic subunit presenilin-1.

Since AD is associated with a progressive impairment of several aspects of cognition, including spatial disorientation and episodic memory failures in human patients and animal models, we next studied the performance of MSG-treated rats in the Barnes circular maze test, a hippocampus-dependent cognitive task that requires spatial reference memory. In this test, MSG-treated rats aged between 6 to 9 months searched significantly higher number of non-target holes than control rats before they identified the target hole (Table 1).

Systemic administration of MSG leads to glutamate-induced cell death in specific brain regions, including hypothalamus and cerebellum. This further results in dysregulation of ghrelin system. Ghrelin is a multi-functional hormone produced in a wide variety of tissues, including brain that stimulates growth hormone release, regulation of food intake, insulin release, pancreatic islet ß-cell survival, adiposity and control of energy homeostasis. Dysregulation of ghrelin system is not restricted to the development of obesity and metabolic syndrome, but is also involved in AD. In fact, it has recently been reported that AD patients have a reduction in local brain ghrelin production, as compared with age-matched controls. Ghrelin has neuroprotective action. Recently, it has been shown that systemic injection of ghrelin ameliorates neurodegeneration in intrahippocampal oligomeric Aβ injected mice.

Ghrelin has also been found to be reduced in obese humans. Indeed, ghrelin receptor analogs are being tested as anti-obesity drugs. It has recently been demonstrated that one of the ghrelin receptor analogs, viz., [D-Lys (3)] GHRP-6 markedly reduces blood glucose and improves related metabolic abnormalities in a mouse model of obesity and reverses the hyperleptinemia seen in obese rats. However, no studies have yet addressed the effect of this compound on AD-related mechanisms.

Given the potent effect of ghrelin in obesity, we evaluated the influence of [D-Lys (3)] GHRP-6 in terms of cognitive deficits, modulation of AChE activity and Aβ levels. It was evident from Fig. 2 that [D-Lys (3)] GHRP-6 was effective in bringing down the elevated glucose levels seen in MSG-treated rats to that of controls (Fig. 2A). Interestingly, [D-Lys (3)] GHRP-6 lowered the hippocampal Aβ levels (Fig. 2B) and serum leptin levels to basal value (Fig. 2C), elevated the ghrelin levels to that of matched controls (Fig. 2D), reduced the activity of AChE (Fig. 2E) and significantly restored the cognitive functions to an appreciable extent (Fig. 2F). These findings demonstrated the therapeutic potential of [D-Lys (3)] GHRP-6 in reversing the AD-like changes observed in MSG-treated obese rat model. It is pertinent to mention at this juncture that [D-Lys (3)] GHRP-6 brought about 10% reduction in the body weight of MSG-treated rats. However, it did not exert any influence when administered to control rats (data not shown).

The present findings strengthened the case for using the MSG-induced obese rat as a non-transgenic experimental rodent model for AD research. The current data showed that the ghrelin receptor antagonist [D-Lys (3)] GHRP-6 could be beneficial...
in the development of AD therapeutics. However, further studies are necessary to establish the close association between ghrelin system and AD.

Acknowledgement

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References


Fig. 2.—Influence of [D-Lys (3)] GHRP-6 on MSG-treated rats. [Shown are the levels of serum glucose (A), hippocampal Aβ (B), serum leptin (C), serum ghrelin (D) and AChE activity (E) in the hippocampal extracts of rats treated either with saline or MSG or MSG, followed by ghrelin antagonist. Data (n = 6) represent mean ± S.D. *P<0.05, **P<0.001 and ***P<0.0001 vs MSG-treated rats. MSG-induced cognitive impairment measured by Barnes maze (F) shows the number of wrong holes searched before identifying the escape tunnel after the training sessions. Data represent mean ± S.D. (n = 6)]
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