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EFFECT OF DIAZEPAM AND BICUCULLINE IN BODY WEIGHT REGULATION: POSSIBLE ROLE OF GABA TYPE A RECEPTOR

Amit Goyal[*1] and Anjoo Kamboj [2]

Department of Pharmacology [1], Chandigarh College of Pharmacy, Landran, Mohali - 140110, Punjab, India.
I. K. Gujral Punjab Technical University [2], Jalandhar, Kapurthala - 144603, Punjab, India.

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Correspondence to Author:
Amit Goyal

Department of Pharmacology, Chandigarh College of Pharmacy, Landran, Mohali - 140110, Punjab, India.

E-mail: cgc.ccp.ag@gmail.com

ABSTRACT: Arcuate nucleus GABAergic system plays an important role in the energy balance evidenced by various studies. The present study was designed to investigate the involvement of GABA type A receptor in experimental obesity. In the present study effects of chronic administration of diazepam (0.5 and 1mg/kg/day, i.p) and bicuculline (0.6 and 1.25mg/kg/day, i.p) specific GABA type A receptor agonist and antagonist respectively for 8 weeks along with high fat diet to the obese rats which were pre-treated with high fat diet feeding for 8 weeks on the various parameters of obesity were analysed. Treatment with bicuculline (0.6 and 1.25mg/kg/day, i.p) produced significant dose dependent decrease ($p < 0.05$) in various parameters of obesity as compared to high fat diet group. Diazepam (0.5 and 1mg/kg/day, i.p) produced dose dependent increase in the feeding behaviour in experimental obese rats, while the other parameters were not significantly altered with chronic administration of diazepam as compared to high fat diet control group. Diazepam a negatively alter the parameters of obesity, while the bicuculline positively modulate the parameters of obesity. The present data demonstrated that high fat diet induced obesity was prevented by GABA type A receptor blocker bicuculline. The diazepam a GABA type A receptor agonist increased the feeding behaviour in rats and promote the fat deposition which in-turn result in obese rats, while this effect was not significantly differ from the alone high fat diet group. These findings give the strong evidence of involvement of GABA type A receptor in obesity.

INTRODUCTION: GABA (γ-aminobutyric acid) has been proposed to play a key role in the regulation of food intake and body weight [1]. Psychological factors, genetic predisposition and eating habits can increase body weight which may lead to obesity [2].

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It is a serious and rising public health burden worldwide; it is no more viewed as a cosmetic issue, but it becomes a potential risk factor of various co morbidities, such as type 2 diabetes, cardiovascular morbidity, cancer [2, 3]. Thus, the obesity epidemic shortens the length and impaired the quality of life of current and future generations, and it presents a significant challenge to future health-care budgets. Obesity is characterized with accumulation of excess fat in body causing adverse affect on health [4, 5]. Obesity occurs when the balance between food intake and energy expenditure is disrupted, *i.e.* more food is

consumed than utilized, leading to excess fat stores being laid down. There are many environmental factors that predispose individuals to gain weight, *e.g.* freely available high-calorie food and sedentary life style. Genetic factors also contribute to this imbalance [6].

In the severely obese, surgical intervention may be necessary. An alternative approach is to develop therapeutic agents that can either reduce food consumption or increase energy utilization. Despite intensive research on obesity pathogenesis, an effective therapeutic strategy to treat and cure obesity is still lacking. Currently, only a few FDA-approved anti-obesity drugs like orlistat, lorcaserin, phentermine-topiramate and naltrexone-bupropion are available in the market, but they have considerable side effects [7]. Exciting studies in last decades have established the importance of neural pathway in the hypothalamus in the regulation of body weight homeostasis. Recent research significantly expanded the list of neurotransmitters involved in body weight-regulating neural pathways [8].

Understanding the function of neurotransmitters released from key neurons for energy balance regulation was essential for delineating neural pathways and eventually for designing effective therapeutic drugs against the obesity epidemic. Considerable efforts had been devoted to the development of weight-control medications that target neurotransmitters in the brain that regulate food intake [9]. Several neurotransmitters (dopamine, GABA, norepinephrine, serotonin) as well as peptides and amino-acids are involved in the regulation of food intake [10].

The cerebral mechanisms underlying the behaviours that lead to pathological overeating and obesity were poorly understood [11]. GABA (γ-aminobutyric acid) a inhibitory neurotransmitter in the mammalian brain mainly present in the arcuate nucleus had been proposed to play a key role in the food preferences of selecting the type, quantity and quality of food by regulating the transmission of signals between neurons in brain circuits [12]. Additionally, the GABA a inhibitory neurotransmitter mainly involved in eating behaviour in experiments using rodent models and GABA receptor agonists and antagonists [13].

GABA acts on two types of receptors: ionotropic $GABA_A$ receptors ($GABA_A$Rs), which are mainly located postsynaptically and metabotropic $GABA_B$ receptors ($GABA_B$Rs), which are located pre-synaptically as well as postsynaptically in the CNS [14, 15]. The role of GABA was extensively explored on pathogenesis of obesity but the involvement of its different receptor subtypes was not well understood. The previous studies demonstrated that $GABA_B$ receptor agonist had body weight reducing effect [16] but the role of $GABA_A$ receptor in body weight regulation had not yet explored.

So the present study was designed to explore the role of GABA type A receptor in obesity by employing its receptor agonist and antagonist *i.e.* diazepam and bicuculline respectively. The present results indicated that $GABA_A$ receptor agonist was having body weight promoting role and blockade of this receptor had preventive role in obesity. Through the use of $GABA_A$ receptor agonist/antagonist the present study reported that $GABA_A$ receptor has major role in obesity.

MATERIALS AND METHODS:

Drugs and Chemicals: Casein (Modern Diary, New Karnal, India) and cholesterol (Thomas Baker, Mumbai, India) were used to prepare high fat diet. Orlistat (S)- 2- formylamino- 4- methyl- pentanoic acid (S)- 1- [[(2S, 3S)- 3- hexyl- 4- oxo- 2- oxetanyl]methyl]dodecyl ester and Diazepam 7-Chloro-1-methyl-5-phenyl-3H- 1, 4-benzodiazepin-2(1H)- one was purchased from Sigma-Aldrich. Bicuculline peripheral GABA type A antagonist was purchased from (Genetix Biotech Asia Pvt. Ltd New Delhi - 110 015 India). All the drugs were dissolved in dimethyl sulfoxide (DMSO; 10%, v/v). The estimation kits for serum glucose, cholesterol, triglycerides and HDL were obtained from (Reckon Diagnostics [P] Ltd. Vadodara, India). All other chemicals used in the present study were of analytical grade. All drug solutions were freshly prepared before use.

High Fat Diet-induced Obesity: Experimental obesity was induced by feeding high-fat diet (containing; powdered normal chow, 365g; lard, 310g; casein, 250g; cholesterol, 10g; Vitamin mix and mineral mix, 60g; dl-methionine, 03g; yeast powder, 01g; sodium chloride (NaCl), 01g were added to make 1.0 kg of diet), to rats [17].

The high fat diet contained 5.33 Kcal/gm while the normal chow contains 3.80 Kcal/gm. This diet provides 68% energy as carbohydrate, 20% as protein and 12% as fat to produce obesity in rats while as normal chow provides 65% of energy as carbohydrate, 20% as protein and 4% as fat [18].

Animal Treatment: Male wistar rats of 7-8 weeks of age were procured from the animal facility of the Institute. The animals were housed in poly-propylene animal cages (two rats/cage) and maintained under controlled room temperature (25 ± 2 °C) with 12:12 h light and dark cycle. The guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India were followed and prior permission was sought from the institutional animal ethics committee (approval no. 1201/PO/RE/S/08/CPCSEA) for conducting the study. Animals were fed with normal chow (NC) or high fat diet (HFD) for 8 weeks. Animals were divided into different groups and each group contain 6 animals. Animals fed on NC were continued on same diet for further 8 weeks and were assigned as group 1.

HFD fed animals randomized on the basis of their body weight and divided into different 7 groups (group 2 to 8) and these groups were continued on HFD for another 8 weeks. Group 2 was not given any treatment and assigned as HFD control. Group 3 was given DMSO 1ml kg^{-1} day^{-1}, i.p [19] and assigned as vehicle control. Group 4 was given orlistat 30mg kg^{-1} day^{-1}, p.o. [19] and assigned as standard control. Group 5 and 6 were given bicuculline 0.6 and 1.25mg kg^{-1} day^{-1}, i.p. respectively [20] and group 7 and 8 were given diazepam 0.5 and 1mg kg^{-1} day^{-1}, i.p. respectively [21]. All the animals had free access to water and the animals were inspected daily. Food intake and body weight were measured twice weekly.

At the end of the stipulated period, blood for various biochemical parameters was obtained by retro-orbital puncture under light ether anaesthesia and the animals were sacrificed by cervical dislocation. The blood was collected into tubes, serum separated and analyzed on the same day. The epididymal, mesenteric and retroperitoneal white adipose tissue (WAT) were dissected, cleaned of, weighed and stored in 10% buffered formalin solution for histological analysis. Lee index [22] *i.e.* (Body Wt in gms)1/3 / (ano-nasal length in cm) an index of obesity, was calculated at the end of the experiment.

Histological Analysis and Morphometry: Epididymal adipose tissue was fixed in 10% formalin and then embedded with paraffin. Tissue sections (10μm) were cut and mounted on microscope slides. After being air-dried, they were stained with hematoxylin and eosin and photographed at 100X magnification. At least two fields per slice and six slices per fat mass were analyzed for the purpose of quantifying adipocyte size.

Measurements: Serum glucose, triglyceride, total cholesterol and HDL cholesterol concentrations were measured by using commercially available kits.

Statistical Analysis: All values are expressed as mean ± standard deviation (STDEV). The significance of the differences between the means of various groups was established by one way ANOVA followed by Tukey's multiple range test using the Graph pad Prism 4 software. The p value < 0.05 was considered to be statistically significant.

RESULTS: Administration of HFD for 8 weeks significantly ($p < 0.05$) increased body weight of animals then the age matched normal control rats and there was no significant difference of body weights of animals between the various treatment groups before initiation of treatment (**Table 1**).

Effect of Various Pharmacological Interventions on Body Weight, Adipose Tissue Weight and Lee Index: Obese rats after 16 week of HFD feeding had significantly increased body weight and total fat content then the age matched normal control rats. Lee index was also significantly increased in obese rats as compared to normal rats. However treatment with bicuculline from 9-16 weeks attenuated HFD induced increase in body weight, adipose pads weight and lee index. Moreover administration of diazepam from 9-16 weeks did not attenuated HFD induced increase in body weight, adipose pads weight and lee index. Administration of dimethyl sulfoxide from 9-16 weeks did not affect HFD induced increase in body weight, adipose pads weight and lee index.

Administration of orlistat a standard drug of obesity from 9-16 weeks produced significant reduction in body weight gain, adipose pads weight and lee index in obese rats (**Table 2**).

TABLE 1: EFFECT OF VARIOUS PHARMACOLOGICAL INTERVENTIONS ON BODY WEIGHT OF ANIMALS AT DIFFERENT TIME INTERVALS

Groups	Initial Body weight	Body weight at the end of 8th week	Body weight at the end of 16th week
NC	222 ± 9.3	242 ± 13.5	282 ± 17.8
OHFD-C	233 ± 8.1	312.1 ± 5.03[a]	399 ± 16.2[a]
Vehicle control (10% v/v DMSO, 1ml kg^{-1})	234.3 ± 7.9	316.3 ± 8.5[a]	397 ± 13[a]
Orlistat (30mg kg^{-1})	236 ± 11.9	309.5 ± 13.6[a]	299 ± 14.6[b]
Bicuculline (0.6 mg kg^{-1})	226 ± 9.2	305.6 ± 9.5[a]	322 ± 16.04[b]
Bicuculline (1.25 mg kg^{-1})	234 ± 9.7	314.1 ± 8.6[a]	309 ± 14.6[b]
Diazepam (0.5 mg kg^{-1})	225 ± 10.48	303.3 ± 9.8[a]	395.8 ± 14.9[a]
Diazepam (1 mg kg^{-1})	229.16 ± 9.7	302.5 ± 9.3[a]	396.6 ± 14.7[a]

All values are expressed as Mean ± Standard deviation (STDEV), n = 6, one-way ANOVA followed by Tukey's multiple range test. DMSO: dimethyl sulfoxide. [a] = $p < 0.05$ *vs.* NC (normal control), [b] = $p < 0.05$ *vs.* OHFD-C (Obese high fat diet control).

Effect of Various Pharmacological Interventions on Biochemical Parameters: Obese rats after 16 week of HFD feeding had higher glucose, triglyceride and total cholesterol level as compared to the age matched normal control rats. However treatment with bicuculline from 9-16 weeks attenuated HFD induced hyperglycemia, hyper-triglyceridemia and hyper-cholesterolemia. Moreover administration of diazepam from 9-16 weeks did not attenuated HFD induced hyper-glycemia, hyper-triglyceridemia and hyper-cholesterolemia. Administration of dimethyl sulfoxide from 9-16 weeks did not affect HFD induced hyperglycemia, hyper-triglyceridemia and hyper-cholesterolemia. Administration of orlistat a standard drug of obesity from 9-16 weeks produced significant reduction in the level of glucose, triglyceride and total cholesterol in obese rats (**Table 2**).

TABLE 2: EFFECT OF VARIOUS PHARMACOLOGICAL INTERVENTIONS ON VARIOUS PARAMETERS IN OBESE RATS

					Bicuculline (mg kg^{-1})		Diazepam (mg kg^{-1})	
Parameters	NC	OHFD-C	Vehicle Control (10% v/v DMSO, 1ml kg^{-1})	Orlistat (30mg kg^{-1})	0.6	1.25	0.5	1
Initial body wt (g)	222±9.3	233±8.1	234.3±7.9	236±11.9	226±9.2	234±9.7	225±10.48	229.16±9.7
Final body wt (g)	282±17.8	399±16.2[a]	397±13[a]	299±14.6[b]	322±16.04[b]	309±14.6[b]	395.8±14.9[a]	396.6±14.7[a]
Lee index	348±9.2	389±16.7[a]	394.19±14[a]	361±21.4[b]	377±14.3	357±13.4[b]	388.8±18.5[a]	397.6±18.6[a]
Feed intake Kcal day^{-1}	92±7.1	111±7.8[a]	110.15±10.4[a]	85±12.8[b]	103±9.9[b]	98±6.2[b]	115.4±8.7[a]	119.9±8.08[a,b]
Epididymal fat	1.75±0.23	5.25±0.94[a]	5.26±0.9[a]	1.90±0.32[b]	2.08±0.36[b]	2.7±0.39[b]	4.35±.42[a]	4.61±.39[a]
Retroperitoneal fat	1.56±0.29	5.8±0.87[a]	5.78±0.89[a]	1.96±0.28[b]	1.96±0.30[b]	1.8±0.28[b]	4.46±.47[a]	4.55±.46[a]
Mesentric fat	2.1±0.19	5.6±0.95[a]	5.71±0.74[a]	2.6±0.38[b]	2.56±0.50[b]	1.9±0.14[b]	4.25±.48[a]	4.45±.45[a]
Glucose (mg dL^{-1})	94±5.5	150.5±6.47[a]	149.6±5.8[a]	96.7±4.09[b]	98±2.75[b]	94.3±1.75[b]	140.6±12.14[a]	146.3±6.47[a]
TG (mg dL^{-1})	67.7±4.4	146.5±10.65[a]	145.5±10.4[a]	71±5.17[b]	73.5±4.08[b]	70.5±5.12[b]	137.8±7.83[a]	143.3±3.55[a]
TC (mg dL^{-1})	95.9±4.1	163±12.64[a]	162.6±12.8[a]	95.8±5.19[b]	96.8±1.94[b]	92.8±5.19[b]	158.3±8.9[a]	151.5±13.4[a]
LDL (mg dL^{-1})	49.8±5.7	110.3±12.54[a]	110.9±11.5[a]	49.4±7.06[b]	50.13±2.4[b]	45.4±6.0[b]	103.1±10.41[a]	96.3±14.13[a]
VLDL (mg dL^{-1})	13.5±0.89	29.3±2.13[a]	29.1±2[a]	14.2±1.03[b]	14.7±0.81[b]	14.1±1.02[b]	27.5±1.57[a]	28.6±0.71[a]
HDL (mg dL^{-1})	32.5±2.19	23.3±2.94[a]	22.6±1.75[a]	32.1±2.85[b]	32±2.44[b]	33.3±1.21[b]	27.6±1.50[a]	26.5±1.87[a]

All values are expressed as Mean ± Standard deviation (STDEV), n = 6, one-way ANOVA followed by Tukey's multiple range test. DMSO: dimethyl sulfoxide, TG: Triglycerides, TC: Total cholesterol, LDL: Low density lipoproteins, VLDL: Very low density lipoproteins, HDL: High density lipoproteins. [a] = $p < 0.05$ *vs.* NC (normal control), [b] = $p < 0.05$ *vs.* OHFD-C (Obese high fat diet control).

Effect of Various Pharmacological Interventions on Daily Feed Intake (Kcal): In high fat diet model, a significant increase ($p < 0.05$) in feed consumption (Kcal) was observed as compared to normal chow fed rats. Orlistat which was standard control in the present study significantly decreases the feed consumption as compared to HFD fed rats. Administration of dimethyl sulfoxide from 9-16 weeks did not affect feed consumption of animals as compared to HFD fed rats. The food intake was significantly decreased by the administration of bicuculline from 9-16 weeks. On the other hand, the food intake was increased by the administration

of diazepam from 9-16 weeks as compared to HFD rats (**Table 2**).

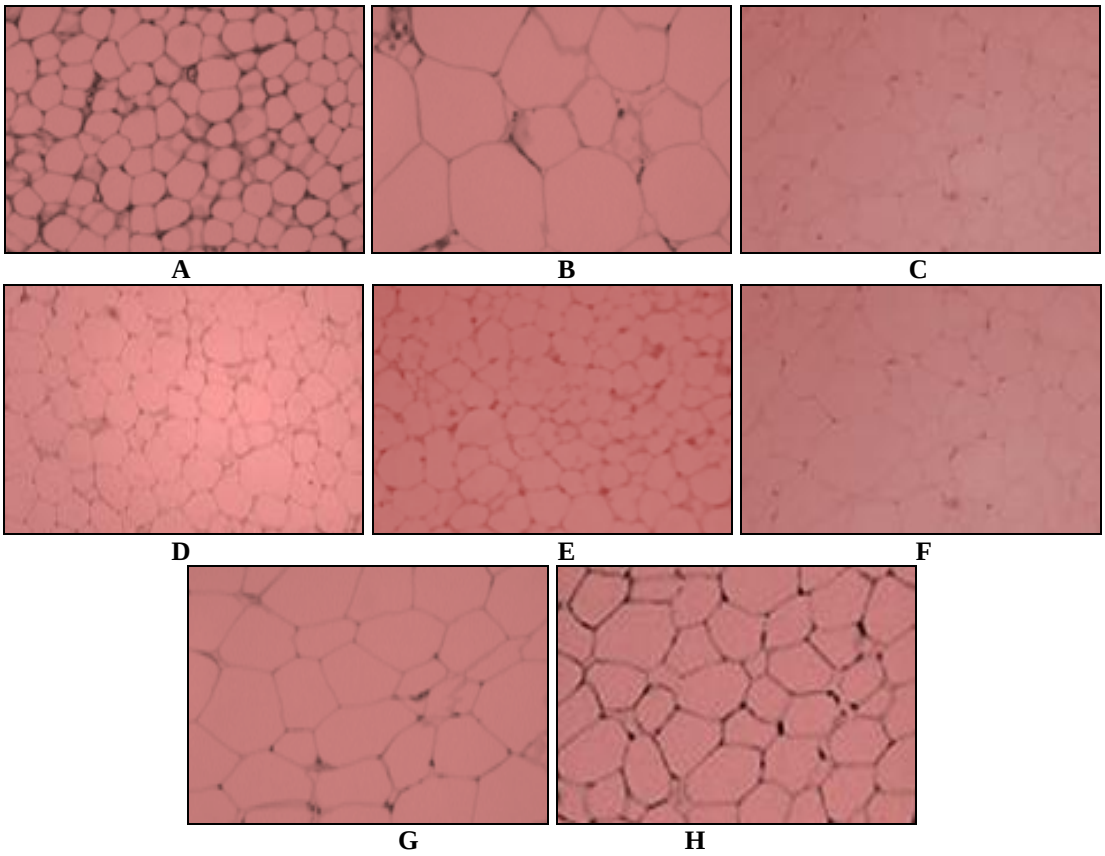

FIG. 1A: EFFECT OF VARIOUS PHARMACOLOGICAL INTERVENTIONS ON ADIPOCYTE SIZE

(A) (a) adipocyte size of normal control animals; (b) adipocyte size of obese high fat diet control animals; (c) adipocyte size of vehicle control animals; (d) adipocyte size of standard control animals; (e) adipocyte size of animals given Diazepam 0.5mg kg^{-1} day^{-1}; (f) adipocyte size of animals given Diazepam 1mg kg^{-1} day^{-1}; (g) adipocyte size of animals given Bicuculline 0.6mg kg^{-1} day^{-1}; (h) adipocyte size of animals given Bicuculline 1.25mg kg^{-1} day^{-1}.

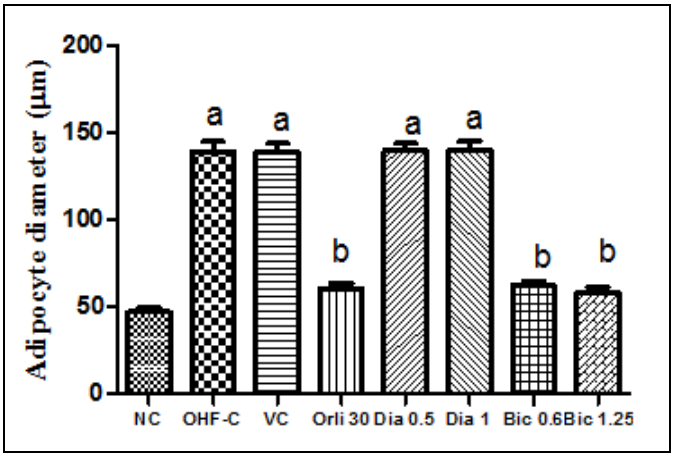

FIG. 1B: AVERAGE ADIPOCYTE DIAMETER OF DIFFERENT GROUPS

All values are expressed as Mean ± Standard deviation (STDEV), one-way ANOVA followed by Tukey's multiple range test. VC: Vehicle control, Dia 0.5: Diazepam 0.5mg kg^{-1} day^{-1}, Dia 1: Diazepam 1mg kg^{-1} day^{-1}, Bic 0.6: Bicuculline 0.6mg kg^{-1} day^{-1}, Bic 1.25: Bicuculline 1.25mg kg^{-1} day^{-1}, Orli 30: Orlistat 30mg kg^{-1} day^{-1} ^{a} = $p < 0.05$ vs. NC (normal control), ^{b} = $p < 0.05$ vs. OHFD-C (Obese high fat diet control).

DISCUSSION: In the present study, experimental obesity was developed by long term high fat diet treatment. The body weight gain observed in the present study is consistent with studies in animal models, suggesting that exposure to high concentrations of carbohydrates or HFD contribute to the development of overweight or obesity [23].

Notably, metabolic disturbance results in elevation of plasma lipids [24] which is characterized by elevated TC, TG levels, LDL-C levels and decreased serum HDL-C [25]. Further, feeding with high fat diet caused hyperglycemia in rats [26]. Therefore the serum lipid levels (total cholesterol, LDL, VLDL, HDL, and triglycerides) and glucose levels were estimated in present study as the marker of hyperlipidemia and hyperglycemia. Hypercaloric diets can modify the GABA levels in the brain. There is a relationship between HFD intake and neurotransmitter concentration in the rat brain [27].

The role of GABA in the regulation of food intake is not well understood. The present study was undertaken to examine the role of GABA in experimental obesity by peripheral administration of GABA receptor modulators, bicuculline and diazepam in two different doses *i.e.* low and high dose. The present data demonstrated that in HFD rats, (1) high dose of bicuculline treatment significantly reduced food intake, Lee index and body weight increase; (2) the weight of WAT was significantly decreased and the biochemical levels of glucose, TG, TC, LDL and VLDL was significantly improved by high dose of bicuculline treatment; and (3) Diazepam in both low and high dose did not affect any parameter of obesity as compared to HFD rats, Moreover feed consumption was significantly increased after administration of high dose of diazepam.

The last finding is of particular interest, since in earlier findings it had been shown that dietary GABA intake decreases the body weight increase in genetically obese mice [28], other previous studies demonstrated that decrease of GABA release from Agrp neurons of GABA knockout mice was lean [29], one study reveals that baclofen a selective GABA type B agonist decreases body weight [16]. However present study shows that diazepam a GABA type A receptor agonist did not decreases the body weight increase in HFD treated obese rats, moreover feed consumption was increased with diazepam administration. It is likely that GABA administered peripherally in the previous studies decreased body weight increase in the genetically obese mice may be due to GABA action on GABA type B receptor, while in present study diazepam a GABA type A receptor agonist does not decrease body weight increase, it confirm that GABA type A is involved in body weight promoting role while GABA type B has body weight reducing effect.

The present study, confirmed these findings by employing bicuculline a GABA type A receptor antagonist, in high doses bicuculline significantly decreases the parameters of obesity. To our knowledge, this is the first study in which the effects of diazepam and bicuculline on diet induced obese rats were examined and our results suggest that the effects of these pharmacological innervations on food intake and body weight were clearly dependent on GABA receptor modulation.

Among peripheral signals related to energy balance, leptin, an adipocyte-derived hormone, plays a critical role in energy balance as evidenced by obesity resulting from a genetic deficiency of leptin (ob/ob mice) or its receptor (db/db mice) [30]. Leptin decreases body weight by decreasing the GABA release from GABA-ergic neurons and disinhibition or stimulation of pro-opiomelanocortin (POMC) [31] expressions in the arcuate nucleus, plasma leptin levels are elevated in obesity probably due to reduced efficacy of brain leptin transport [32] or desensitization of its receptors [33] and desensitization of leptin receptor result in elevated GABA level. Thus, postsynaptic GABA especially GABA type A receptor blockade by its antagonist could be an alternative treatment for obesity.

One study showed that food restriction increases the GABA release from Agrp. The GABA action on POMC neurons decreases energy expenditure and increase obesity, so by this correlation we can say that GABA mimetic action increases body weight while its antagonistic action decreases body weight, thus bicuculline block GABA action mainly through GABA type A receptor thus prevent obesity. Present study also suggest that there is less involvement of GABA type B receptor in obesity as GABA type B agonist baclofen block GABA release through presynaptic GABA stores and decreased level of GABA in turn prevent obesity and body weight reduction in case of baclofen treatment was not due postsynaptic GABA type B receptor.

Furthermore, Diazepam a GABA type A agonist, increased food intake in HFD treated rats, increase in food intake in the present study were due to GABA type A receptor mimetic action of diazepam on POMC neurons which in turn decreases POMC activity. In this study, bicuculline decreased the weight and size of adipocytes of HFD fed rats. These changes in adipocytes possibly increased secretion of adiponectin from adipose tissues [34], leading to further improvement in glucose tolerance [35]. Present data demonstrated that GABA type A antagonist bicuculline has the potential to work downstream of POMC receptor and thereby reduce food intake and increase energy expenditure in obese rats.

CONCLUSION: On the basis of above discussion, it may be concluded that GABA type A receptor has role in the body weight regulation. The GABA receptor modulation by its receptor agonist / antagonist alters the various parameters of experimental obesity. Bicuculline attenuated HFD-induced increase in the body weight, visceral adipose pad weights, and lee index, serum TC, TG, and glucose levels. The present results suggesting that GABA type A receptor antagonist could be new therapeutic reagent for obesity. These findings give the strong evidence of involvement of GABA type A receptor in obesity.

Ethical Approval: The guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India were followed and prior permission was sought from the institutional animal ethics committee (approval no. 1201/PO/RE/S/08/CPCSEA) for conducting the study.

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CONFLICT OF INTEREST: Author declares no conflicts of interest.

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